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Title of Dissertation: "Pregnancy Specific Glycoprotein 17 Binds to the Extracellular Loop 2 of its Receptor, CD9, and Induces the Secretion of IL-10, IL-6, PGE_2 and $TGF\beta_1$ in Murine Macrophages"

Name of Candidate: Cam Ha
Doctor of Philosophy Degree
9 July 2004

Dissertation and Abstract Approved:

M L Cutler — 7/9/04
Mary Lou Cutler, Ph.D. — Date
Department of Pathology
Committee Chairperson

[signature] — 8/7/04
Gabriela Dveksler, Ph.D. — Date
Department of Pathology
Committee Member

Susan R Haynes — 7/9/04
Susan Haynes, Ph.D. — Date
Department of Biochemistry
Committee Member

[signature] — 7/9/04
Tao-Yiao John Wu, Ph.D. — Date
Department of OB/GYN
Committee Member

[signature] — 07/09/2004
Carl Dieffenbach, Ph.D. — Date
National Institutes of Health
Committee Member

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Cam Thi Ha
Graduate Degree Program in Molecular and Cell Biology
Uniformed Services University of the Health Sciences

ABSTRACT

Title of Dissertation:	Pregnancy Specific Glycoprotein 17 binds to the extracellular loop 2 of its receptor, CD9, and induces the secretion of IL-10, IL-6, PGE_2, and $TGF\beta_1$ in murine macrophages.
Name, degree, year:	Cam Thi Ha Doctor of Philosophy 2004
Dissertation directed by:	Gabriela S. Dveksler, Ph.D. Associate Professor Department of Pathology Uniformed Services University of the Health Sciences

Pregnancy specific glycoproteins (PSGs) are a family of proteins produced by primate and rodent placentas and secreted into the maternal circulation. In humans PSGs are detected in maternal serum from the time of implantation, and their concentration increases when pregnancy progresses, reaching 200-400 microgram/ml at term. The correlation between low levels of PSGs and poor pregnancy outcomes suggests PSGs play a role in the regulation of maternal immunity to the semi-allogeneic fetus.

We have previously reported that human and murine PSGs induce anti-inflammatory cytokines in monocytes, and identified CD9 as the cellular receptor for murine PSG17. However, the role of CD9 in cytokine induction mediated by PSG17 as well as the signaling mechanism triggered in macrophages in response to PSG17 remain largely unknown. The present research attempted to shed light on these two areas.

Protein-protein interaction assays were employed to examine the direct interaction between the two molecules, showing that the N1 domain of PSG17 (PSG17N) bound directly to the second extracellular loop of CD9 (CD9-EC2). FACS and ELISA revealed that phenylalanine174 in CD9-EC2 was essential for PSG17 binding.

Treatment of C57BL/6 macrophages with PSG17N induced IL-10, IL-6, PGE_2, and $TGF\beta_1$ in wild type but not in CD9-deficient cells. These findings suggest that CD9 is the only biological receptor for PSG17N in the induction of these cytokines. In addition to PSG17, PSG19 was found to use CD9 as its receptor. Treatment of macrophages with PSG17N in the presence of a COX-2 specific inhibitor significantly reduced the level of IL-10 and IL-6 secretion. Furthermore, co-treatment with PSG17 and a cAMP-dependent protein kinase A inhibitor significantly decreased the production of IL-10 and IL-6. These two findings implicate the involvement of the COX-2 mediated PGE_2 pathway in cytokine induction.

In conclusion, the expression of CD9 is required for the induction of IL-10, IL-6, PGE_2, and $TGF\beta_1$ by PSG17N in murine macrophages, while COX-2/ PGE_2 and cAMP-dependent/PKA are involved in the up-regulation of IL-10 and IL-6. These discoveries enhance our understanding of the functions of PSGs and support the hypothesis that PSGs are involved in the regulation of the maternal immune response in pregnancy.

PREGNANCY SPECIFIC GLYCOPROTEIN 17

BINDS TO THE EXTRACELLULAR LOOP 2 OF ITS RECEPTOR, CD9, AND

INDUCES THE SECRETION OF IL-10, IL-6, PGE_2, AND $TGF\beta_1$

IN MURINE MACROPHAGES

by

Cam Thi Ha

Dissertation submitted to the Faculty of the
Graduate Degree Program in Molecular and Cell Biology of the
Uniformed Services University of the Health Sciences
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
2004

ACKNOWLEDGMENTS

First of all, I wish to express my appreciation for the guidance and encouragement that were given to me by Professor Mary Lou Cutler – Chair of my Dissertation Committee, and by Dr. Carl W. Dieffenbach, Professor Susan Haynes and Professor Tao-Yiao John Wu.

I am most grateful to Professor Gabriela Dveksler, my Major Advisor. Ever since our first encounter at the Uniformed Services University Professor Dveksler has been a great teacher who provides me with excellent learning opportunities, a mentor who gives me good counsel on being a scientist, and a great model for creativeness, leadership, and other personality traits that I want to acquire and cultivate.

For research materials and advice on investigatory techniques, I remain indebted to: Dr. Claude Boucheix of Institut André Lwoff, Hopital Paul Brousse (France); Dr. Shoshana Levy of the Division of Oncology, Stanford University School of Medicine; Dr. Paul Primakoff of the Department of Cell Biology and Human Anatomy, University of California Davis; and Dr. Wolfgang Zimmerman of the Tumor Immunology Laboratory, University Clinic Grosshadern, Ludwig Maximilians University (Munich).

I am also most appreciative of the friendship and assistance I have received from my classmates Michelle Crawford, Lillian Gray and John Pesce, and from my co-workers in Dr. Dveksler's Pathology Laboratory, especially, Diego Ogando, Roseann Waterhouse, Jennifer Wessells, Kim White, Julie Wu and Carolyn Zalepa.

And for my husband, Lawrence Newton, who had not only the fortitude to read through innumerable drafts of my dissertation but also the forbearance to sit through innumerable rehearsals of my dissertation defense, I will carve in stone a big Thank You.

To My Mother
Who Always Believes in Me

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GLOSSARY

AA: arachidonic acid

APC: antigen presenting cell

CD9KO: knock-out CD9

CD9WT: wild-type CD9

CEA: carcinoembryonic antigen

CEACAM: CEA-related cell adhesion molecule

CSF-1: colony stimulating factor 1

CTB: cytotrophoblast

CTL: cytotoxic T lymphocyte

dNK: decidual natural killer cell

EAE: experimental autoimmune encephalomyelitis

EC1: extracellular loop 1

EC2: extracellular loop 2

EVT: extravillous trophoblast

hCG: human chorionic gonadotropin

HCS: placenta cell line

HCSf: HCS-derived suppressor factor

hPGH: human placenta growth hormone

IgSF: immunoglobulin superfamily

IDO: indoleamine 2,3-dioxygenase

IVF/ET: in vitro fertilization/embryo transfer

LIF: leukemia inhibitory factor

MBP: myelin basic protein

M-CSF: macrophage-colony stimulating factor

MIP: macrophage inflammatory protein

PGDH: prostaglandin dehydrogenase

PI4K: Phosphoinositide 4 kinase

PKA: protein kinase A

PKC: protein kinase C

PSG: pregnancy specific glycoprotein

RGD: arginine-glycine-aspartic acid

RSA: recurrent spontaneous abortion

SFQ: serine-phenylalanine-glutamine

STB: syncytiotrophoblast

TCR: T cell receptor

Treg: regulatory T cell

uNK: uterine natural killer cell

PART ONE

INTRODUCTION

1 THE CEA FAMILY AND ITS CEACAM AND PSG SUBGROUPS

The carcinoembryonic antigen (CEA) family, which is comprised of two subgroups, CEA-related Cell Adhesion Molecule (CEACAM) and Pregnancy Specific β Glycoprotein (PSG), belongs to the large Immunoglobulin superfamily (IgSF) [1].

In humans, 28 identified CEA/CEA-related genes are clustered at chromosome 19q13.2 [2]. CEA gene members share a special organization, which is used as a criterion for member determination. Each gene is composed of different numbers of exons. In all members of the family the first exon codes for a leader-like peptide (about 20 amino acids) and the second exon codes for the first N-terminal domain (or N-domain) of the mature protein. This N-domain resembles the immunoglobulin variable portion of an Ig molecule, whereas the other exons individually code for the Ig-constant-like domains [3]. At least 11 pseudogenes of the CEACAM subclass have been identified, but none in the PSG subgroup [4].

CEACAM gene members display a high conservation in their N-terminal domain (>90% nucleotide similarity) and in their promoter sequences, even between human and rodent genes while N-domains of CEACAM genes share only 65-75% similarity with those of the PSG subgroup [5]. CEACAM is comprised of two kinds of membrane bound proteins: proteins of one kind are anchored to the cell surface through their transmembrane domains while proteins of the other are linked to glycophosphatidylinositol (GPI) lipid moieties. Unlike the CEACAMs, most PSG members are secreted proteins [4]. All proteins in the CEA family are highly glycosylated, and the carbohydrate portion may account for 30-60% of their protein mass [6].

The time of divergence between the CEACAM and PSG subgroups was estimated to be around 107.7 ± 17.1 million years ago [7]. Nowadays, these subgroups maintain a 60% sequence similarity while performing different functions that are cell-type specific. The CEACAM subgroup is associated with a wide spectrum of biological properties including intercellular cell adhesion [8], signal transduction [9], microbial phagocytosis [10], and tumor progression such as colorectal tumor [8] and lung cancer [11]. In addition, several investigators have established the role of this subgroup in the regulation of innate and adaptive immunity [12-15]. Meanwhile, PSGs have been linked to pregnancy complications and fetal pathologies [16] [17], and have been shown to induce cytokine secretion *in vitro* [18, 19]. Specifically, the biological functions of many CEA members have been found to be mediated through the binding of their N-domains to putative ligands or receptors [18-21] [22].

2 HUMAN PSGs: STRUCTURE, EXPRESSION, AND FUNCTIONS

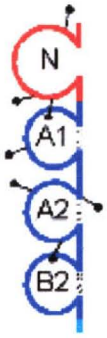
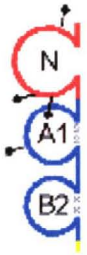
2.1 Structure and domain organization of PSG genes and proteins

Eleven PSG genes (*PSG* 1-11) are located in a tandem array on chromosome 19q13.2 within the CEA family region [23, 24]. All PSG gene members contain the same exon order, i.e., L, L/N, A1, B1, A2, and B2. Following the first exon L, which contains the 5' untranslated region and the first 21 amino acids of the leader peptide, the L/N exon encodes the rest of the leader peptide and the Ig-variable-like (IgG-V like) domain of approximately 110 amino acids. Exons A and B encode Ig-constant-like domains (IgC-like), containing, respectively, 92-93 and 85 amino acids. There are at least two cysteine

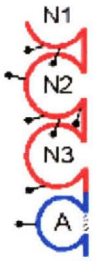
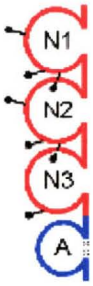
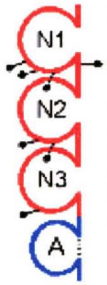
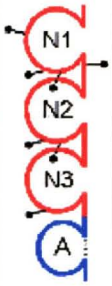
residues in each Ig-C like domain, and the disulfide bridge built by these cysteines is assumed to stabilize the immunoglobulin structure [23].

Due to alternate splicing, mRNAs of PSGs are present in various forms. More than 28 splice variants have been identified to date. While the N-terminal domain is present in every transcript, the composition of the individual molecules is determined by the number of Ig-C-like domains as well as by the number and identity of the A or B subtypes that are retained. For instance, the domain structure is categorized as L-N-A1-A2-B2-C (type I), L-N-A1-B2-C (type IIa), L-N-A2-B2-C (type IIb), as well as a number of unusual shorter forms [25]. Noticeably, alternate splicing of the region at the 3' end of PSG genes results in varying numbers of transcripts, which, once expressed, form a set of identical proteins that differ only in the length of their C-terminal domains (3-21 amino acids). As a result more than 37 different gene products are all co-expressed during pregnancy. The cDNAs of PSGs share 90% sequence homology, whereas at the protein level the homology is 79% [3]. (Figure1A)

PSGs isolated from human placentae have different sizes, including 110, 68-94, and 41 kDa species [26, 27]. Using *in vitro* translation of placental poly(A) + RNA, Watanabe and Chou have characterized several nonglycosylated proteins of 50, 48, and 36 kDa [28]. The high molecular weight (MW) observed for native PSGs isolated from placental extracts appears to be the result of a glycosylation that occurs at 4-8 potential N-linked glycosylation sites [26, 27]. Therefore, carbohydrate accounts for at least 30% of protein mass for PSGs [29]. Our lab has confirmed that recombinant PSGs produced in bacteria have a lower MW than those synthesized in insect and mammalian cells (Dveksler, personal communication).

protein (old name)	PSG1-4C1 (PSG1a)	PSG2-3C5 (PSG2n)	PSG3-4 (PSG3)
domain organization (largest variant)			
gene (old name)	*PSG1*	*PSG2*	*PSG3*
accession no. used		M20882	M23575
splice variants	5	1	1

1A: Human PSGs

PSG16 (bCea)	PSG17 (Cea2)	PSG18 (Cea3)	PSG19-4 (Cea4a)
			
Psg16 (bCea)	*Psg17* (Cea2)	*Psg18* (Cea3)	*Psg19* (Cea4)
U34272	M83337	M83346	
1	1	1	2

1B: Murine PSGs

= external link

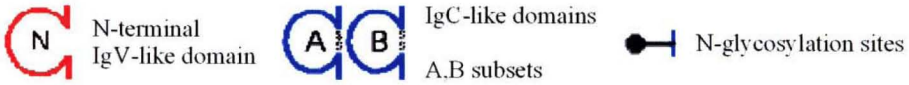


Figure 1. Representation of domain organization of human and murine PSGs. Whereas murine PSGs have at least two N-terminal IgV-like domains but only one C-terminal IgC-like domain, human PSGs (PSG 1-11) have only one N-terminal Ig-like domain followed by 2 or 3 C-terminal IgC-like domains.

(Source: http://cea.klinikum.uni-muenchen.de/)

Generally conjugated to the core protein by nitrogen (N-linked) or oxygen (O-linked), the cell-specific carbohydrate portion (glycan) can perform its function in cell adhesion and signaling modulation dependently or independently of the protein core [30]. The precise role of post-translational modifications in PSGs has not been studied but preliminary results from our laboratory indicate that glycans on PSG17 are required for binding to its receptor, CD9.

2.2 PSG synthesis in placenta—The regulation of gene expression

PSGs were discovered in pregnant human serum more than 30 years ago [31] and later identified in human placenta [29]. Using specific antibody analysis with BAP-3, which was raised against the B2 domain, Zhou et al. revealed the exclusive expression of human PSGs in syncytiotrophoblasts (STBs) isolated from first- and second-trimester and term placentae. In addition, PSGs have been identified in all three cellular compartments responsible for processing secreted protein, namely rough endoplasmic reticulum, the Golgi complex, and secretory vesicles [32]. The fact that PSGs are synthesized by STB, the outermost layer of the placenta that aggressively invades the uterine wall during placentation, suggests a role for the PSG family in structural modulation at the fetomaternal junction.

It has been reported that mRNAs of all human PSGs are present in placenta at different levels, although it is difficult to determine the protein expression pattern because of high mAb cross-reactivity [23, 33] [34]. Recently, using DNA microarray analysis, Aronow identified PSG1 as the most active transcript up-regulated (70 fold) during the *in vitro* cell differentiation of cytotrophoblasts (CTB) to syncytiotrophoblasts [35]. The expression of PSGs is regulated mainly at the transcription level and increases

along with placental development [36]. The promoter regions of PSGs are 90% conserved and lack a TATA box, a pyrimidine rich initiator element, and a G+C rich pattern [23] [37]. While the class I genes (i.e. PSG1 and 3) contain an imperfect binding site for SP1, a ubiquitous transcription factor, the class II genes (PSG 5, 6,11) possess a perfect SP1-binding sequence that acts as a cis-regulating element. Recently, Nores et al. reported that the SP1 protein is co-expressed with PSGs in the STB cells, and is able to activate PSG5 promoter constructs in transfection experiments, suggesting its potential role in the up-regulation of PSG expression *in vivo* [38]. Furthermore, there is evidence that the down-regulation of PSGs in both placenta and non-placenta cell lines is controlled by various proximal or distal repressor elements located within the 5' flanking region of the PSG genes [39] [40] [38].

2.3 Association of PSG production with pregnancy outcome

PSG release starts 3-4 days after fertilization, coincident with blastocyst implantation [41]. The proteins are secreted from the placenta to the maternal circulation and detected as early as 7 days post implantation. The serum level of these proteins increases with gestation progression and reaches up to 200-400 μg/ml at term, far exceeding the concentration of human chorionic gonadotropin (hCG) and alpha fetoprotein [42, 43].

Low concentration of PSGs is correlated with certain pregnancy complications, including recurrent spontaneous abortion (RSA) [16], pre-eclampsia [44], fetal hypoxia [45], intrauterine growth retardation [17, 46-48], or fetal congenital defects [49]. Abnormally high levels of PSGs are used as clinical markers of trophoblastic hyperplasia, e.g., hydatidaform mole, and nontrophoblastic disorders, e.g., breast cancer [50] [51].

The contribution of PSGs to the maintenance of normal pregnancies is demonstrated by *in vivo* experiments using anti-PSG antibodies in monkeys and mice. Administration of anti-PSG antibodies results in spontaneous abortion and an impaired fertility in non-pregnant females [52] [53]. According to Arnold, PSG11 induces IL-10 in human monocytes, and the expression of PSG11 in the endometria of women who suffer unknown RSA is significantly low [54]**.** Interestingly, in a study of rheumatoid arthritis patients, women who have high PSG levels experience an improvement in their condition during their pregnancy that is not observed in those lacking a heightened level of PSGs [55]. Taken together, these findings suggest an immunologically protective role for PSGs in maintaining a successful pregnancy.

2.4 Functions of PSGs

Studies using recombinant PSGs have begun to shed light on the functions of these proteins. One feature of particular interest is the conserved motif of arginine-glycine-aspartic acid (RGD) residues present in the N-domain of PSG2, 3, 5, 6, 7, 9, and 11. This sequence is identified in several ligands of integrins, such as fibrinogen and vitronectin, whose binding to integrins promotes cell migration and proliferation [56]. Using a 20 amino acid synthetic peptide containing the RGD residues of PSG11, Rutherfurd reported that this sequence mediates the binding to putative receptors that are present on myeloid cells but not on T or B cells, suggesting a role for RGD-bearing PSGs in the binding to maternal immune cells [57]. However, PSG1, the most abundant form as measured by DNA microarray analysis [35], lacks this motif. So far there is no functional analysis to confirm this speculation.

The discovery that PSGs induce cytokines in human and murine macrophages has led to the consideration that human PSGs may function as a component of the immuno-modulatory machinery of maternal immunity. Treatment of monocytes and macrophages with PSG 1, 6, and 11 induces anti-inflammatory cytokines including $TGF\beta_1$, IL-10, and IL-6, but not pro-inflammatory cytokines such as $TNF\alpha$, IL-1β, and IL-12, suggesting that PSGs exert an influence on cytokine polarization in pregnancy [19] [54]. These effects have been shown to be mediated by the N-domain of PSGs [19] [18]. Recently, Motran et al. have reported that *in vivo* expression of PSG1a induces alternate activation of monocytes, and enhances a Th2-type immune response. In particular, treatment of monocytes with PSG1a *in vitro* induces the activation of arginase, a contributing factor to alternative activation of macrophages that subsequently suppresses cell-dependent T cell proliferation [58, 59].

It has been reported that PSGs may also be produced by cells other than placental cells. For instance, human PSGs are expressed in the non-pregnant state including breast cancer, peripheral blood cells, and bone marrow cells [51] [60] [61]. However, the exact roles of human PSG in these ectopic sites remain largely unclear. One study indicates that in non-pregnant sera the concentration of PSGs is very low, approximately 0.5 μg/L [62]. Another study establishes that high expression of PSGs in breast cancer is correlated with a poor prognosis [60]. Additionally, Blomberg et al. reported that mice that had been given a specific dose of PSG experienced a recovery of platelet and white blood cell counts following bone marrow transplantion [61], which seems to indicate that PSGs have played some role in such recovery.

3. MURINE PSGs

3.1 Murine PSG structure and expression

Besides humans, PSGs have been detected in primates [63], rats [64], and mice [65]. There are 15 murine PSG genes (*psg16-30*) which are located in the centromere-proximal region of chromosome 7 [65]. Chromosome 7 is syntenic to human chromosome 19 [66] where PSG genes are present [67]. All murine PSG genes have been partially or fully sequenced. Like other members of the CEA family, murine PSG genes are comprised of varying numbers of exons, which encode individual Ig-like domains. Generally, murine PSGs have one to two splice variants which contain varying numbers of IgV-like domains (N1-N5) and only one IgC-like domain (A), which is absent in PSG30 (CEA website)[*]. Murine PSGs share 54-93% sequence homology at the amino acid level [65]. Ruderd and coworkers report that the first IgV-like domain of most PSGs (N1 domain) has a partly conserved sequence that corresponds to the RGD motif on human PSGs. The motif is described as having 4 residues in alternative orders as R/H-G-E/K, in which the glycine is always present [65]. Similar to human, murine PSGs are highly glycosylated with 6-11 potential N-linked glycosylation sites. However at the amino acid level the N1 domains of murine and the N-terminal domain of human PSGs do not have high similarity (CEA website)*. (Figure 1B)

Murine PSG transcripts are detectable by means of RT-PCR analysis in the placenta and the pooled tissues of embryo but not in adult tissues, including kidney, lung, testis, ovary, liver, brain, thymus, heart, and spleen (tested for PSG17, 18, and 19), as reported by Kromer and coworkers [68]. *In situ* hybridization detects the expression of

[*] http://cea.klinikum.uni-muenchen.de/

murine PSGs by primary and secondary trophoblast giant cells early in gestation and later, primarily, by spongiotrophoblasts [68]. Moreover, PSG transcripts are found in mouse placenta beginning at day 6.5 of gestation, or 2 days after implantation [18]. RNase protection assays reveal that the expression of murine PSGs increases during embryonic development (E) and reaches the maximal level at E15.5-17.5, indicating a coordinated expression of splice variants during placental and embryo development [68]. However, the concentration of murine PSGs in maternal blood remains undetermined due to the unavailability of methods to measure these proteins in serum.

3.2 Murine PSG functions

Human PSGs (1, 6, and 11) have been shown to induce anti-inflammatory cytokines in human and mouse macrophages [19]. To determine whether murine PSGs mimic such an effect in mouse macrophages, Wessells conducted a study of PSG18 expression and function. It showed that PSG18 induced the up-regulation of IL-10 at the levels of mRNA and protein expression in macrophages. Furthermore, PSG18 did not induce the production of the pro-inflammatory cytokines IL-1β, TNFα, and IL-12 and the inflammatory mediator iNOS. As expected, the N1-domain of PSG18 was sufficient to induce IL-10, just like other truncated forms of human PSGs tested [18]. Additional support for the view that PSGs function as Th2 promoting molecules comes from our recent work showing that murine PSG17 induces IL-10, IL-6, and TGFβ_1 in both primary and immortalized macrophage cell lines [69].

Our laboratory has determined that CD9 is the receptor for PSG17 by panning, and ELISA and FACS analysis were used to confirm this result [21]. Since CD9 is the first receptor identified for a member of the PSG family, several questions arose at this

juncture of our investigation: Is CD9 the only receptor for PSG17, is it involved in the observed cytokine induction, and is CD9 the receptor for other members of the murine and human PSG family? My research is an attempt to address these questions.

Recently, PSG17 has been shown to regulate antigen presenting cell (APC)-dependent T cell proliferation and function in an myelin basic protein specific T cell receptor (MBP-specific TCR) transgenic mouse model, which is highly susceptible to experimental autoimmune encephalomyelitis (EAE), an autoimmune disease-induced condition (Dveksler, personal communication). Together with the PSG1-mediated inhibitory effect on T cell proliferation discussed earlier [59], this suggests a wider role for PSGs in the immuno-modulation process which includes both the innate and adaptive immune systems.

4 CD9 AND TETRASPANINS

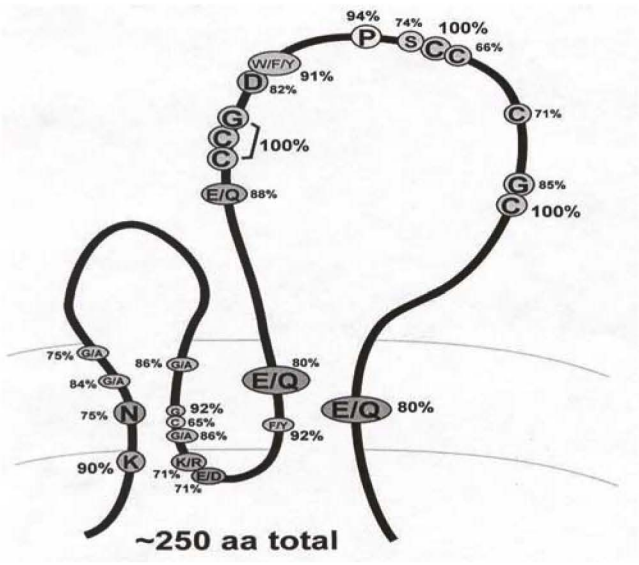
4.1 Tetraspanins

Tetraspanins are membrane-bound glycoproteins of 25-50 kDa. They were discovered in the early 1990's, and first identified as leukocyte cell-surface proteins. Later they were found to be expressed in several mammalian cell types such as hematopoietic and non-hematopoietic cells, excluding red blood cells, as well as in mammalian tissues [70-72]. Tetraspanin genes have been mapped to different chromosomes in humans and mice. So far, at least 30 members of the tetraspanin family have been identified in mammals [73].

Tetraspanins are characterized by the presence of four conserved transmembrane domains that are flanked by two extracellular loops of different sizes and

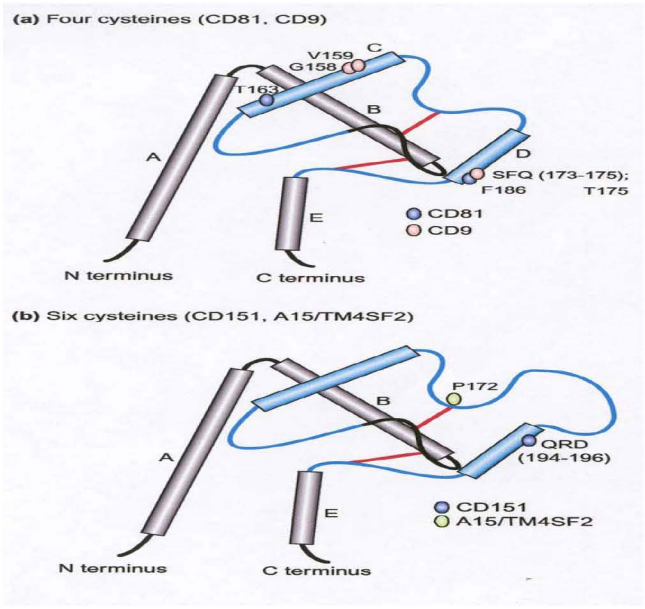
one small intracellular loop. The first small extracellular loop (EC1) contains 20-27 amino acids, whereas the second (EC2) is larger and has around 75-130 residues. The two N and C terminal tails are both cytoplasmic and contain only 5-14 residues. While the four transmembrane domains are highly conserved, the extracellular domains show variation in length, residue arrangement, and degree of glycosylation. The most conserved feature of the EC2 is the cysteine containing motifs (Figure 2A). Together with polar residues within the transmembrane domains, the cysteine motifs help to distinguish tetraspanins from other four-transmembrane domain-containing molecules [70] [73, 74].

The least conserved domain is the EC2, which is comprised of two sub-domains—constant and variable—in some tetraspanin members (such as CD9, CD81, CD151, and A15), as reported by Stipp (Figure 2B). While the constant regions are suggested to be a possible site for homodimerization of tetraspanins, the variable regions have been shown to be essential for specific ligand-receptor engagement [75] [76]. The association between tetraspanin members, which is crucial for the formation of the tetraspanin complex, is mediated by hydrophobic interactions involving the transmembrane domains. Despite being such small parts of the molecule, the cytoplasmic tails of some tetraspanins have gained some recent attention. For example, it was reported that the mutation of the GYEVM motif in the CD63 C-terminal tail renders the molecule incapable of translocating to the intracellular compartment, and, as a result, causes it to travel to the cell surface. Also of great interest is the finding that cysteine residues located in the tetraspanin transmembrane domain adjacent to the cytoplasmic side of the membrane and in N- and C-cytoplasmic tails are targets of palmitoylation.



2A. Representation of conserved residues in most tetraspanins

(Source: Hemler M., *Journal of Cell Biology* 155:1103-7, 2001)



2B. Subdomain structure of some tetraspanin's large extracellular loop 2. While the constant region (gray) is suggested to be the place for homo-dimerization, the variable sub-domain has been shown to mediate specific protein-protein interaction.

(Source: Stipp C. et al., *Trends in Biochemical Sciences,* 28: 106-117, 2003)

Figure 2. Structure of tetraspanins

Palmitoylation is a post-translational modification that adds palmitate, a 16-carbon fatty acid, to the thio group of a cysteine residue through the thioester linkage created by the CxxC motif. As a result, this process can modulate protein-protein interaction or target the modified proteins to membrane microdomains [77]. Palmitoylation of cysteine residues, hence, may be regarded as a potential mechanism in the establishment of lateral protein associations [71, 73].

While like most cell surface molecules tetraspanins lack enzymatic domains, various antibody engagement experiments have shown that they participate in diverse cellular activities, such as proliferation, adhesion, migration, and activation. It is believed that these activities take place as a result of the extraordinary ability of tetraspanins to incorporate various molecules into their complex. Upon organizing a multi-molecular network, tetraspanins work as a bridge that brings cell-surface molecules closer to their putative signaling partners or, contrarily, act as a barrier that keeps these molecules apart. As a result, in the formation of the tetraspanin web, specific stimulation can trigger or prevent specific signaling pathways [73, 78].

In immune cells the incorporated proteins often found in multi-molecular tetraspanin complexes include integrins, immunoreceptors, the EWI family, and signaling molecules [78, 79]. The interaction between tetraspanin members, believed to be through their transmembrane domains, is essential for the formation of the web, which in turn promotes the incorporation of other interacting molecules. In general, the interaction of molecules in the tetraspanin web is classified into three categories, ranging from a primary interaction that is direct, robust, and highly resistant to detergents (level 1) to

secondary ones, which are less stoichiometric and maintained only in weak detergents (levels 2 and 3) [78].

4.2 CD9, a member of the Tetraspanin Superfamily

CD9 is a 24-27 kDa glycoprotein expressed abundantly in various tissues, hemapoietic as well as non-hemapoietic, including human extravillous cytotrophoblasts [80] [81]. The dense presence of CD9 in T cells, monocytes, DC, marginal zone B cells, plasma cells, and human decidual NK cells [78, 82-84] hints at a potential role in immunoregulation.

CD9-associated molecules have been found to include $\alpha_{(3-4)}\beta_1$ integrins, PKC, PI4K, other tetraspanin members (CD81, CD82, CD63, CD53), and various immune system-specific receptors (CD2, CD5, CD4, CD8, CD28) [78]. Remarkably, EWI-F and EWI-2, which are type I transmembrane proteins of the Ig superfamily, have a robust and specific interaction with CD9 or CD81 [85, 86]. While the expression of EWI-2 is observed in almost all cell types, it is more restricted for EWI-F, whose function includes the negative regulation of $PGF_{2\alpha}$ [87].

Currently, most functional studies of CD9 are performed by means of anti-CD9 antibodies to target effector cells, or via the observation of the effect of over-expression of CD9 on cellular activities. CD9 functions, for instance, have been linked to activation of signaling molecules. As reported by Kaji and co-workers, the treatment of macrophages with anti-CD9 induces the co-cross-linking of CD9 and FcγRs, which in turn activates macrophages as a result of syk phosphorylation [88]. In addition, by associating with PI 4-kinase and transmembrane-bound TGFα, CD9 has been found to be involved in mediating cell growth, survival, and activation as reported in various cell

types including epithelial and hematopoietic cell lines [89] [90]. There is evidence that CD9, by recruiting protein kinase C (PKC) to the vicinity of integrins, can promote the phosphorylation of PKC, leading to cell differentiation and proliferation [91].

There are indications that CD9 also plays a role in the regulation of immune responses. According to Vogt, on APCs CD9 and some tetraspanins, such as CD63, CD81 or CD82, are capable of incorporating antigen-engaged MHC class II complexes, leading to an enhanced antigen-presenting ability of these cells in the absence of antigen-specific T cell interaction [92]. Additionally, in the co-stimulation with a suboptimal dose of CD3, anti-CD9 antibodies have been shown to activate T cells independently of a CD28-costimulatory signal [82].

Studies of CD9-deficient mice, while not showing severe abnormality of the aforementioned biological processes, revealed that egg-sperm fusion is defective. The residues SFQ 173-175 of the EC2 of CD9 on the eggs are essential for the gamete fusion. Therefore, lacking CD9 on the egg renders the females infertile but has no effect on males [93] [76]. It has also been suggested that the fusion of gamete membranes requires the interaction of CD9 in the eggs with its binding partner(s), either in *cis* or *trans*.

So far, PSG17 is the only soluble ligand identified for CD9. The different methods used for this discovery involve the examination of the PSG17N binding to CD-9 expressing cells. ”Do these two molecules interact with each other directly?” and “Where is the PSG17 binding site on CD9?” are some of the follow-up questions that need answering. My present research aimed at providing these answers.

5 EARLY EVENTS IN HUMAN FETAL DEVELOPMENT AND SELECTED PLACENTA-ASSOCIATED DISORDERS: AN OVERVIEW

Human development is a continuous process that starts with fertilization; a union of paternal and maternal gametes resulting in a zygote, progresses through a fetal growing period, and ends with the successful delivery of a viable fetus. The hallmarks of a recognized conception are the two synergetic events of placentation and decidualization, which are necessary for embryo attachment and development.

First, the blastocyst, resulting from zygote cleavage, proceeds to attach to the uterine wall and subsequently invades the endometrial epithelium and underlying connective tissue. Then at gestation day (gd) 10, and as a result of an aggressive invasion mediated by its trophoblasts, the conceptus, comprised of embryo and associated membranes, is completely planted in the endometrium. Meanwhile, in response to increased levels of progesterone, the stromal cells of the uterus are transformed into lipid- and glycogen-filled decidual cells in a process known as decidual reaction, which is also essential for embryo implantation. Together the decidual layer and the placental membrane, which is derived from trophoblasts, constitute the maternal and fetal part of the placenta, respectively. Embryonic development ends at the end of 8 weeks, followed by a fetal developing period during which tissues and organs continue to grow and differentiate until parturition.

Functionally the placenta is an endocrine organ that synthesizes various hormones (i.e., progesterone, chorionic gonadotropin, and placental lactogen) and secreted factors such as prostaglandins and cytokines that are essential for maintaining a suitable environment for pregnancy and fetal development. In addition, while the

placenta is a place where nutrients, hormones, oxygen, metabolites, and immune agents are exchanged actively and extensively between mother and fetus, it is also a special structure (known as haemochorial placenta) where maternal circulation comes into direct contact with fetal tissues. As a result, fetal homeostasis is subjected to molecular and cellular change in maternal circulation, noticeably at the early stage of pregnancy [94-96]. (Figure 3)

Gestational and placental disorders can affect not only the outcome of a pregnancy, i.e., fetal well being, but also contribute to the morbidity of mother and fetus [94] [97]:

- *Spontaneous abortion* with a nonviable fetus (termed *abortus* if it weighs less than 500 g), accounts for 50% of threatened abortion which is a common complication in 25% of all pregnancies and still a significant cause of intrauterine and prenatal morbidity. Normally, a pre-term fetus stands a good chance of survival if it is delivered after 22 weeks. *Recurrent spontaneous abortion* is defined as having three or more abortions before the 20^{th} week of gestation. A *missed abortion* refers to a condition in which the uterus still keeps a nonviable fetus before a forced or natural abortion occurs.
- *Hydatidaform mole*, an aberrant growth of the placenta accompanied by partly or completely deficient fetal development, can initiate a local or systemic and even cancerous illness for the mother.

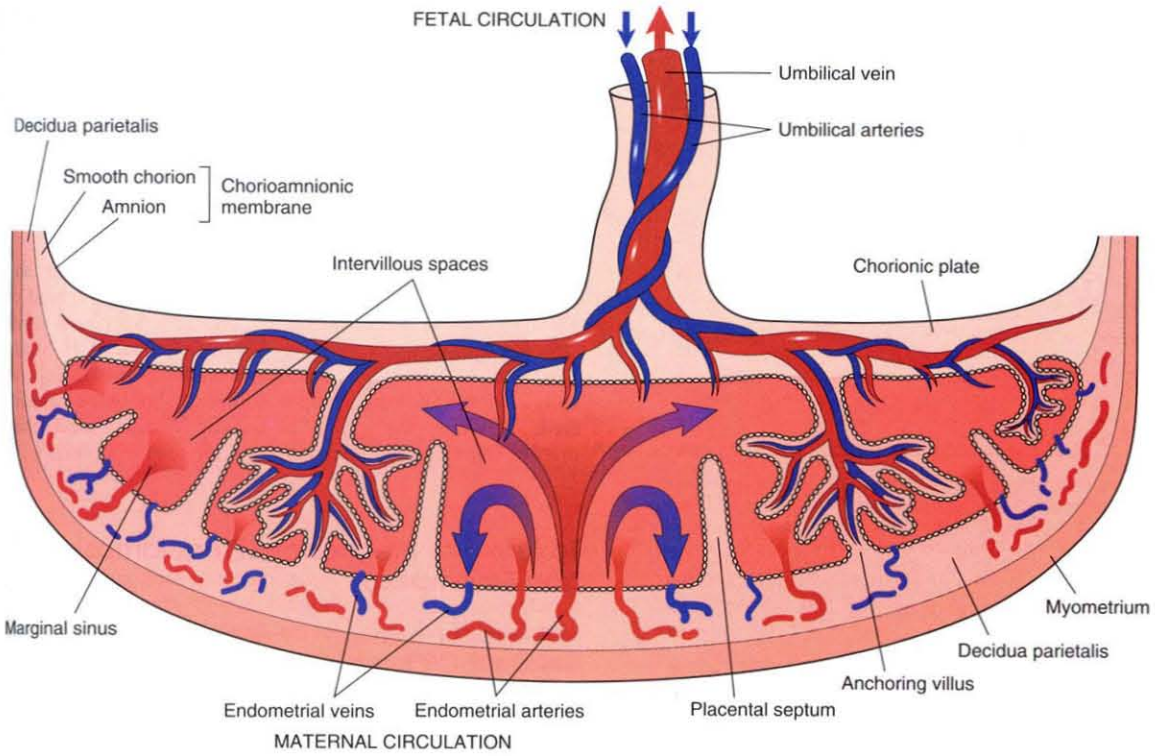


Figure 3. Diagram of full-term placenta. The cytotrophoblastic shell lining with syncytio- and cyto-trophoblastic cells holds in check maternal blood that is circulated by endometrial arteries and veins. Umbilical arteries bring oxygen-poor blood to the placenta while umbilical veins transfer the blood rich with nutrients and oxygen back to the fetus. The placenta is where nutrients, oxygen, metabolites, and immune agents are exchanged extensively between mother and fetus and also a location where maternal circulation comes into direct contact with fetal tissue**.** (Source: Cotran et al. 1999. *Robbins Pathologic Basis of Life*. Sixth Edition. W.B. Saunders Company, PA, p.1081)

- *(Pre)-Eclampsia*, also known as toxemia of pregnancy, often occurs during the third trimester in 6% of pregnant women. Placental ischemia, its key factor, underlies a systemic life-threatening disorder for the mother and concurrent fetal loss in severe cases.

While chromosome abnormality, endocrine malfunction, and uterine structural irregularity account for the majority of pregnancy loss, immune dysfunction is increasingly recognized as another likely cause for some of the gestation- and placenta-associated disorders mentioned above.

The successful embryo is the one that has passed the screening and successfully conditioned the uterus into a state known as "pregnancy recognition". It now faces what appears to be a great challenge to its own survival: How can it develop in the supposedly incompatible immune environment of the mother? The challenge is more apparent than real, however. In reality, the mother, while seldom able to accept organs donated by her children, is generally capable of providing a nurturing haven for the fetus.

6 MECHANISMS RELEVANT TO MATERNAL TOLERANCE OF THE SEMI-ALLOGENEIC FETUS: "IS THE CONCEPTUS AN ACTIVE COMPONENT IN FETAL TOLERANCE DURING PREGNANCY?"

Almost 50 years have passed since Medawar first proposed his theory of "fetal-allograft," in an attempt to explain why and how a fetus, which carries immunogenic antigens inherited from the father, can escape the attack from maternal immunity. Part of his theory suggests that the failure of fetal rejection in the course of a pregnancy is the result of a suppression of the maternal immune system coupled with an impaired ability

of the fetus to present its antigenic components to the mother's immunity. [98]. Findings to date run counter to part of this hypothesis. Maternal immune defense has been shown to be effective in mounting both innate and adaptive immune responses to fight against certain infections, and fetal cells are capable of provoking alloreactions from the mother [99]. These observations raise the possibility that the conceptus, operating as an active immuno-regulating component, competently generates a diverse protective mechanism that gives rise to a healthy pregnancy during its interaction with the maternal immune system [100]. The following mechanisms help to characterize the maintenance of a healthy pregnancy as a joint undertaking of mother and fetus.

6.1 Functions of trophoblasts as an immuno-regulatory component of maternal immunity

The dynamic change of the trophoblast structure during gestational progression is essential for placental and fetal development. The differentiation of trophoblasts into cytotrophoblasts (CTBs) and syncytiotrophoblasts (STBs) correlates with implantation. By proteolytic action STBs actively invade the endometrial stroma, preparing the site for embryo implantation, and create a nutrition source by digesting degenerated decidual cells. During the third trimester, nuclei of multinucleated STBs are aggregated to form syncitial knots, which frequently are broken and carried to the maternal blood. On the other hand, CTBs are capable of proliferating and continuously migrating to the multinucleated mass of STBs, subsequently constituting the outer layer of the cytotrophoblastic shell, which acts as a barrier separating the fetus from its mother tissue (Figure 3). Findings to date indicate that trophoblasts actively modulate maternal immunity and, in some cases, function like members of the innate immune system.

The first piece of evidence supporting an immuno-regulatory function for the trophoblasts comes from the discovery, reported by Kovats and coworkers, of high expression of HLA-G in human trophoblasts [101]. HLA-G is a nonclassical (also termed class Ib) MHC Class I molecule found mainly in the extravillous cytotrophoblasts that invade the decidual layer and, as a result, come to direct contact with maternal tissues. It is widely accepted that the inhibitory effect on NK, CD4+, and CD8+ T cells begins with the binding of HLA-G to its multiple receptors. Consequently, the cells become less cytotoxic and/or unable to generate an immune attack, which implies that when this interaction happens at the fetomaternal interface, it promotes fetal tolerance [102] [103]. In addition, Horuzsko has shown that HLA-G transgenic mice tolerate skin allograft better than control mice, and also suggested that the T cell inactivation in this case may be the result of a compromised maturation of myelomonocytic precursors that is mediated by an overexpression of HLA-G [104]. Recent research revealed the association of HLA-G reduced expression with pre-eclampsia, which is histologically characterized by shallow trophoblastic invasion and placental abnormal vascularity [105].

Support for the view that trophoblasts promote pregnancy success also comes from the finding that murine and human trophoblasts preferentially produce indoleamine 2,3-dioxygenase (IDO) which catalyzes tryptophan degradation [106] [107]. It has been suggested that the absence of tryptophan results in maternal T cell suppression and decreased T cell proliferation, whereas blocking this effect with anti-IDO antibodies induces fetal loss in mice [106] [108]. The immuno-suppressive role of IDO is confirmed in the study by Gorczynski and his coworkers, which shows the contribution of IDO-dependent immune suppression to the success of allograft transplantation. This reaction is

mediated by CD200, a tolerance promoting molecule, via its inactivation of dendritic cells [109]. Their finding implies that the same mechanism may govern host-graft reaction and pregnancy. However, a recent discovery that IDO-deficient mice are able to reproduce at a normal rate and produce litters of standard sizes points to the existence of a network with redundant factors that helps maintain pregnancy in mammals [110].

Furthermore, it has been reported that trophoblasts act as a component of maternal immunity during infection. For instance, in cases of systemic infection with *Listeria monocytogenes*, trophoblasts stimulated with colony stimulating factor 1 (CSF-1) specifically produce neutrophil chemoattractants (KC) and macrophage inflammatory protein (MIP) 2. As a result, lymphocytes recruited by those products effectively limit the infection at the fetomaternal junction [111]. Additionally, HCS-derived suppressor factor (HCSf), produced by a placenta cell line (HCS), has a potent inhibitory effect on T cell proliferation *in vitro* and *in vivo* experiments, again implicating the active role of the placenta in the regulation of maternal immune response [112].

6.2 Th2 bias in pregnancy: A collaboration of innate and adaptive immune responses

At the fetomaternal interface, fetal and maternal tissues including the immune cells of the innate and adaptive immune systems produce both subclasses of cytokines—Th1- and Th2-types. The temporary change in cytokine profile is gestational-age dependent, and sometimes in pregnancy complications, the demarcation between the causes and effects of these cytokines is fuzzy. In particular, many cytokines have been shown to exert both pro- and anti-inflammatory functions, depending on their engagement with their receptors, or on the nature, intensity, and duration of the

stimulation. However, there is compelling evidence of Th2-type cytokine polarization at the fetomaternal interface, a process that plays an essential role in maternal recognition and the maintenance of pregnancy. The following section focuses on how the innate and adaptive immune responses interact in the creation of a Th2-like environment in pregnancy.

6.2.1 Roles of trophoblasts

Schafer-Somi reported that STBs spontaneously secrete IFN-γ within 5 days of *in vitro* fertilization before implantation, whereas at a later stage of pregnancy, CTBs and decidual cells produce IL-10 and IL-4 [113]. This is consistent with the report from Lin et al. that supernatants from cultured fetal placenta tissue isolated from all gestational stages contain these cytokines [114]. Additionally, trophoblast cells also synthesize TNFα, IL-1β, IL-8, and IL-6 [115-119].

Similar to macrophages that are present abundantly in the uterus, trophoblasts display several specific markers of phagocytes, including the expression of CD4, CD14, and IgG-receptor (FcR) [120]. In the study of the human placenta conducted by Goodwin and his coworkers, IL-10, IL4, and $TGF\beta_1$ have been shown to inhibit term placenta cells from releasing PGE_2 in response to IL-1β and TNFα stimulation [121]. Neutralizing anti-$TGF\beta_1$ antibodies stimulate trophoblast proliferation, which implies that $TGF\beta_1$ has a controlling role in trophoblastic invasion in the first trimester [122].

6.2.2 Roles of macrophages/dendritic cells

As a central part of the host's innate defense, classical activated macrophages exhibit exceptional strength in the destruction of infectious and intracellular pathogens. This process is carried out by means of several inflammatory mechanisms including

phagocytosis, apoptosis, nitric oxide production, Th1-type cytokine production, and antigen-specific activation of T cells. Recent published studies suggest that, besides those macrophages called M1 that exhibit Th1-like responses, there exists another subclass of macrophages, called M2, that display immune suppressive properties. M2 function is associated with a high production of $TGF\beta_1$ and other Th2-associated cytokines and is accompanied by a reduced NO synthesis following LPS and IFNγ exposure. The down-regulation of NO in this case is a result of $TGF\beta_1$ elevation and/or the induced activation of arginase, which competes with iNOS for their common substrate, arginine. As a result, arginase-dependent arginine catabolism depletes the substrate for the iNOS pathway and reduces its killing effect, which is restored with arginine supplementation [123] [124]. Additionally, deprivation of arginine has a negative effect on T cell proliferation, indicating a role for arginine in regulating immune response [58, 125, 126]. This immune state of alternative activation also holds true for dendritic cells, a potent APC. For instance, IL-10 treatment of immature dendritic cells has been shown to prevent DC maturation, which in turn inhibits antigen-specific differentiation of naïve T cells. This entire process reflects an impact on the down-regulation of graft vs. host reaction [127] [128].

There is a growing body of evidence for the existence of alternatively activated or suppressor macrophages/DCs at the fetomaternal unit. Macrophages/DCs isolated from placental and uterine tissues express a significant amount of TNFα, IL-10, and $TGF\beta_1$ and low level of IL-1β [129] [130] [131]. Moreover, naïve $CD4^+$ T cells, when primed with decidual DCs, preferentially differentiate into Th2 cells, a process that is inhibited with IL-12 treatment[130]. Heikkinen suggests that a high endogenous production of IL-

10 in human decidual macrophages, which comprise 20% of the haematopoietic mononuclear cells lining the uterus, is effective in blocking the differentiation of these macrophages into DCs. The resulting anti-inflammatory environment at the fetomaternal junction, therefore, helps to explain the delayed appearance of uterine-infectious symptoms [131].

6.2.3 Roles of T lymphocytes

A discussion of the central role of T cells can begin with a quick review of how maternal/host immunity is orchestrated to generate different resolutions for host vs. graft situations in the cases of pregnancy and allograft transplantation. Mellor and Munn proposed a stepwise and collaborating mechanism that, while promoting graft rejection, is able to prevent it from happening to the allogenic fetus [132] (Figure 4).

This view is supported by the following observations:

First, there is strong evidence that Treg (regulatory T cell) or Th2 lymphocytes are the predominant subtypes of T cells resident in or recruited to decidual and endometrial tissues. The infiltration of $CCR4^+$ Th2 cells into human decidua at early pregnancy in response to its chemokine TARC, a product of trophoblasts and endometrial gland cells, suggests a modulation role in immune response for Th2 cytokines secreted by these cells at the fetomaternal unit [133]. Also, according to Nagaeva et al., $TCR\gamma\delta^+/CD56^+$ cells, which undergo maturation in decidual tissue of early pregnancy and account for 50% of uterine lymphocytes, exclusively express IL-10 and TGFβ transcripts [134]. This finding has led to a speculation on the protective effect of these cells, which have been found in increasing number in maternal peripheral blood [135].

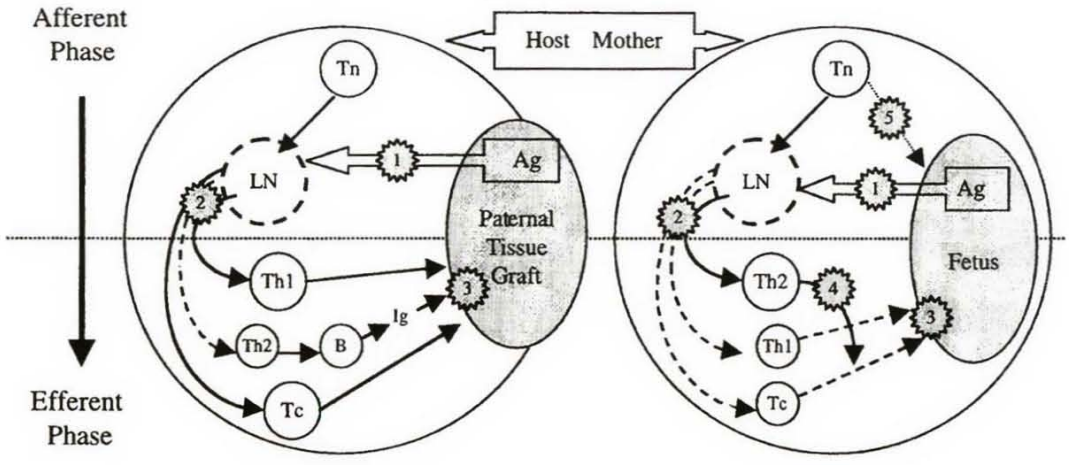


Figure 4. Immuno response of T cell in tissue allograft rejection and in the state of the fetus in pregnancy. Step 1 refers to antigen presentation of host/maternal APCs in draining lymph nodes, whereby naïve T cells (Tn) (also called Th0) are primed and differentiated into Th1 and Th2. These cells then enter circulation (Step 2) and they can perform their specific functions, assisting B cells in the production of antibodies (Th2 effect) or synthesizing pro-inflammatory cytokines (Th1 effect). The combined effects of such performances along with the cytotoxicity of Tc (cytotoxic cells) finally cause a destructive immune attack on allogenic cells (Step 3) and the subsequent rejection of the graft. In pregnancy, there are two additional steps. Step 4 represents a suppression of Th1 effect and Tc activation by Th2 counteraction, where such counteraction is significantly enhanced in the pregnant state. In Step 5, naïve T cells of the mother at the feto-maternal junction have direct access to fetal antigens and, under the influence of various pregnancy-specific factors, may be differentiated into regulatory T cells that have immuno-suppressive property. (Source: Mellor A.L. and Munn D.H., *Annu. Rev. Immunol.*18: 367-391, 2000)

Two mechanisms, direct and indirect immune suppression, have been proposed to explain why there is local intrauterine tolerance to the fetus when these immuno-suppressive cells are prevalent [134] (Figure 5).

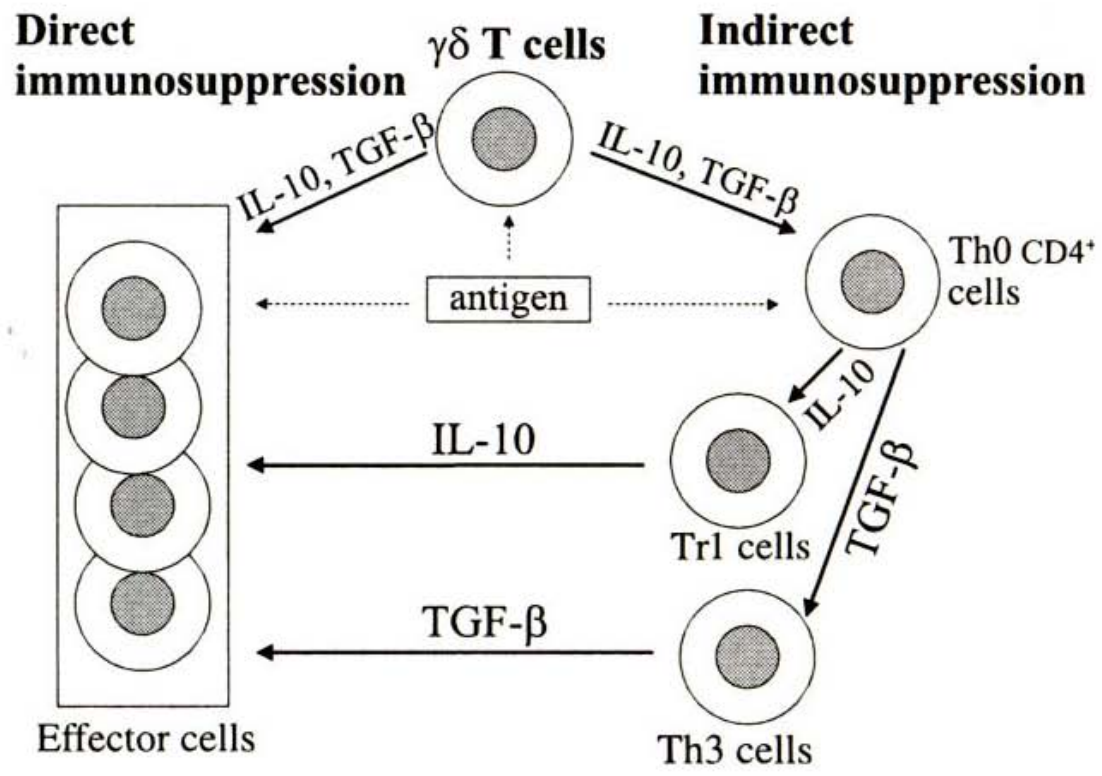


Figure 5. Representation of two possible mechanisms of how decidual γδT cells could induce local intrauterine tolerance to the fetus. Under the influence of pregnancy-associated antigen(s) decidual γδT cells start producing IL-10 and TGF-β. These cytokines could directly inhibit the effector cells (direct immuno-suppression). On the other hand, IL-10 and TGF-β could create a cytokine environment for generation of Tr1 and/or Th3 cells, which in their turn secrete IL-10 and/or TGF-β and suppress the effector cells (indirect immuno-suppression). (Source: Navaega et al., *Am J Reprod Immunol*, 48: 9-17, 2002)

Second, the cell-type shift to Treg and Th2 lymphocytes is also observed in the peripheral blood of normal pregnant women. Research to date also shows in blood of pregnant women the number of IL-4- and IFNγ-secreting T cells increases along with gestation progression. Although the IL-4:IFNγ ratio is unchanged over time, IL-4 produced by these cells dominates the effect of IFNγ, and, by keeping this Th1 cytokine in control, it is able to moderate the Th1 response [136]. Additionally, evaluation of placenta-derived antigen activation of PBMCs indicated that cells derived from women having successful pregnancies show a Th2-prominent reactivity while cells from women experiencing recurrent spontaneous abortion (RSA) displays a high Th1 response [137]. Interestingly, 75 % of women who suffer from rheumatoid arthritis, an autoimmune disease caused by a Th1-dominated destruction of many organs, experience a temporary remission in the course of their pregnancy [138]. In addition, $CD4^+$ $CD25^+$ regulatory T cells, which are essential for allograft survival, are elevated in normal pregnant serum, and these cells suppress the Ag-specific proliferation of T cells that occurs in response to allogeneic DCs [139]. Altogether, these studies confirm that maternal immunity is an active state and, via cytokine selective production, has a significant influence in pregnancy resolution.

Third, Th1-type cytokines are found incompatible with pregnancy since increased levels of these cytokines are detrimental to fetal development as well as pregnancy outcome. A more convincing piece of evidence is the abortion effect of LPS challenge in mice, which is mediated through cytotoxic- and apoptosis-inducing nitric oxide [140]. Studies have confirmed the role of pro-inflammatory cytokines such as IL-2, IFNγ, and TNFα in the termination of pregnancy in rodents [141] [142]. Studies of CD200 (OX-2),

a mediator of tolerance signaling, reveal that this molecule is expressed in human placenta and, when cultured with a mixture of allogeneic lymphocytes, provokes a strong inhibitory proliferation of cytotoxic T lymphocytes (CTL) and causes a bias toward a Th2 reaction [143]. Treatment of pregnant mice with anti-CD200 antibody consistently induces abortion, which can be prevented by infusion with CD200 immuno-adhesin [109]. Placentas collected from malaria-infected pregnant women display an elevation of IFNγ, IL-2, and TNFα, and neonates delivered from these mothers have significantly lower weight compared to those delivered from healthy mothers [144].

Fourth, a polarization toward Th2-type reaction can be employed to keep the Th1-promoting immune response in check, as mentioned previously. Down-regulation of Th2 cytokine synthesis is linked to threatened abortion [145], spontaneous abortion [146] [147], and pre-eclampsia [148] in humans, and fetal resorption in rodents [149]. The failure of implantation after *in vitro* fertilization/embryo transfer (IVF/ET) is associated with an imbalance in Th1 vs. Th2 immune response, in which the number of TNFα-expressing T cells is higher in the implanted subjects than in their normally pregnant counterparts [150]. In stress-triggered abortions using the mouse model reported by Arck et al., the increased ratio of TNFα:TGFβ$_2$ correlates with a higher rate of fetal absorption [151], pointing to a protective function for Th2-associated cytokines. Additionally, in a recent study using this model, uterine $CD8^+$ T cells have been shown to mediate the progesterone by altering the Th1/Th2 cytokine profile via an increase in Th2-type expressing cells [152].

Finally, there is evidence that, in response to progesterone, peripheral lymphocytes of pregnant women produce a distinct molecule known as progesterone-

induced blocking factor (PIBF), which can alter the cytokine profile and suppress lymphocyte activation and NK cell cytotoxicity [153]. Studies in mice reveal that neutralization of PIBF causes an increase in TNFα, a reduction in IL-10, and induces pregnancy loss, which is reversed by administration of anti-NK antibodies [154]. In addition, Szekeres-Bartho has reported that in women who have undergone spontaneous abortion, the low expression of PIBF on lymphocytes is related to the high TNFα level in sera [155]. The recent successful cloning of PIBF promises a new diagnostic tool as well as a therapeutic application for these findings [151].

6.3 Immunologic and vascular modulation of decidual natural killer cells

In immunologic surveillance the primary function of natural killer (NK) cells is to destroy microbe-infected cells by secreting cytokines and releasing cytolytic products [156]. In humans, while peripheral NK (pNK) cells comprise 15% of blood lymphocytes in circulation, decidual NK (dNK) cells account for 70% of total lymphocytes at the maternal interface, suggesting a role in pregnancy [157] [158].

The differential expression of NK cell receptors (such as NKG2, activating or inhibitory killer cell Ig-like receptor KIR, and the immunoglobulin domain receptor CD16), their associated molecules (CD94), and the cell adhesion molecule CD56 is associated with varying degrees of cytolysis by the NK cell subsets. The most powerful killing effect is observed in $CD56^{dim}$ pNK cells that highly express NKG2, KIR, CD16, and cytotoxic granules containing perforin, and granzyme B [84]. Human dNK cells ($CD56^{bright}$ $CD16^{-}$) are a unique subset with immuno-modulatory potential. Using microarray analysis, Koopman and co-workers found that these cells express a wide variety of cell surface molecules, including integrin galactin-1, progestaren-associated

protein 14 (PP14), integrin subunits, multiple tetraspanins (i.e., CD9, CD151, and CD53), and several KIRs, all of which are believed to have a role in immune modulation. In addition, dNK cells have a high content of granules that are believed to be a potent front-line maternal defense mechanism that protects the fetus from viral infection [84].

Murine and human uterine NK (uNK) cells also have a role in remodeling uterine vascularity. Resident in the non-pregnant uterus, uNK cells are immature, inactive, and express predominantly the inhibitory receptor subset. With pregnancy, under the influence of IL-15 and IL-18 associated with the decidualization process, uNK cells undergo maturation and terminal differentiation. Activated uNK cells mainly produce IFNγ that induces gene transcription in uterine stromal cells (a precursor of decidual cells), vascular smooth muscle cells, and the endothelial cells of the spiral arteries. This alteration of the gene expression results in the regulation of placental invasion as well as promotes the dilation of supplying vessels for the placenta in a gestational age specific manner [159-161].

6.4 B lymphocytes and asymmetric IgG antibody production in pregnancy

B cells mediate immunity via their antibody-producing function. The binding of antibodies to the surface of pathogens in extracellular space causes the neutralization, opsonization and/or destruction of the pathogens, which are widely regarded as effective arms of the defense mechanism. However, the observed up-regulation of IL-6 during pregnancy and the ability of IL-6 to promote the production of a subclass of antibodies, known as asymmetric glycosylated IgG antibodies, hint at a protective role for these antibodies in pregnancy. According to Margni and Zenclussen, asymmetric IgG antibodies, while accounting for a small fraction in sera of healthy persons, are produced

in increasing amounts in pregnancy and remarkably, 80% of them show a paternal-derived specific affinity. The protective role in pregnancy of this class is based on their non-precipitating effect on antigens as compared to the precipitating effect of classical immunoglobulins. Since both of these IgG classes compete for the same antigens, changing their ratio will have an impact on antigen-specific destruction. It has been established that in sera from women who experience recurrent spontaneous abortion, the percentage of asymmetric IgG is lower than that from normal pregnant counterparts, 15-18% and 38-47%, respectively [162]. Notably, the antigen non-aggregating effect of asymmetric IgG is reversed by high doses of IL-6, indicating that a tight control in IL-6 synthesis is required to maintain a healthy gestation [163]. The contribution of IL-6 mediated asymmetric IgG needs further evaluation.

In summary, the protective response of maternal immune system toward the allogeneic fetus appears to be a consequence of an interplay of various immunologic factors that is tightly controlled by both the innate and adaptive immune responses.

6.5 Other factors in fetal tolerance

This section presents a brief review of a number of major mechanisms for tolerance.

There is evidence that leukemia inhibitory factor (LIF), which is produced by the endometrium and decidua, promotes implantation and placental growth upon binding to blastocysts during placentation, and to syncytiotrophoblasts at a later stage in gestation [100]. On the other hand, a defective synthesis of LIF by decidual T cells is correlated with RSA [146]. LIF-deficient female mice are fertile but fail to establish blastocyst placentation [164].

A complement inhibitory mechanism in pregnancy is shown to effectively block the antibody-induced complement cascade that otherwise would kill placental cells. Further evidence of such a mechanism comes from the study of Crry-deficient mice. Lacking a complement regulatory factor, these mice die *in utero*, indicating that a complement activation attack on placenta has taken place [165].

Hormones like progesterone and human placental growth hormone (hPGH) that are produced mainly by the placenta, have a strong immune suppressive properties by promoting the production of potent Th2 cytokines such as IL-4. Together with IL-4 these hormones induce T cells at the fetomaternal interface to synthesize LIF and M-CSF, thus maintaining embryo and fetal development [166].

Mechanisms that can inhibit T cell activation or induce T cell anergy are believed to be crucial for fetal tolerance. It is well documented that certain B7 family members can, via ligation to their inhibitory receptors such as CTLA-4 and PD-1, render naïve T cells anergic and suppress T cell activation. Petroff and coworkers recently discovered B7-H1 molecules, which are expressed in trophoblasts but not in placental and maternal macrophages. Thus, it is suggested that upon binding to its inhibitor receptor PD-1, B7-H1 can generate a suppressive effect on intrauterine T cells, thereby enhancing maternal tolerance of the fetus [167].

7 TGFβ_1, IL-10, AND IL-6 IN IMMUNITY AND PREGNANCY

7.1 Transforming growth factor β_1

TGFβ has pleiotrophic effects in regulating the immune response of T cells, B cells, DC, and macrophages. For example, while TGFβ promotes inflammation by

inducing Fcγ RIII expression and increasing IL-1 and TNFα production, it can down-regulate TGFβ receptors or inhibit T lymphocyte proliferation, NK cell activity, lymphokine activated killer (LAK) and cytotoxic T lymphocyte generation, and B cell proliferation [168] [169]. As evidence of the role of TGFβ in immuno-suppression, the administration of TGFβ suppresses symptoms of certain experimentally induced autoimmune diseases whereas the administration of anti -TGFβ antibodies exacerbates these conditions [168]. In addition, Zeller et al. have shown that $TGF\beta_1$ and IL-10 have an additive effect in rendering $CD4^+$T cells hypo-responsive to alloantigens, indicating a mechanism for immune tolerance [170].

The functions of TGFβ in pregnancy have been seen in the remodeling of maternal tissues (e.g., decidualization, apoptosis), cytokine and hormone production, embryonic development, and fetal tolerance [171]. In particular, $TGF\beta_1$ induces cytotrophoblastic cells to produce fibronectin that facilitates the attachment of trophoblasts to the uterine wall during implantation [172, 173]. Together with IL-4 and IL-10, $TGF\beta_1$ has been shown to inhibit the production of IL-1 and TNFα-induced PGE_2 in term human placenta [121]. The importance of this molecule in embryo development is demonstrated clearly in $TGF\beta_1$-deficient mice. More than 50% of $TGF\beta_1$ -/- embryos die between E 9.5-E10.5 because of severe defects in vasculogenesis and haematopoiesis. Live-born mice from $TGF\beta_1$-deficient parents develop a wasting syndrome and die at 3-4 weeks of age due to a pronounced multifocal inflammation [174, 175]. Together, they indicate that TGFβ plays an essential role in maintaining normal immune response.

7.2 Interleukin-10

IL-10 is a potent anti-inflammatory cytokine produced mainly by macrophages, DCs, and regulatory T cells, and is regarded as a macrophage deactivating factor [176]. The anti-inflammatory and immune suppressive properties of IL-10 result from blocking the synthesis of the Th1 promoting cytokine IL-12, down-regulation of a number of macrophage functions. These include phagocytosis, nitric oxide production, and pro-inflammatory cytokine release (i.e. TNFα) [177], and antigen presenting ability [178]. It is interesting to note that in an immune privileged condition called ACAID (anterior chamber-associated immune deviation) the introduction of certain antigens into the anterior chamber of the eyes fails, due to a suppression of Th2 response, to provoke a delayed-type hypersensitivity. This down-regulation is determined by TGFβ_1-mediated secretion of IL-10 during antigen presentation [179]. Gene-targeted deletion of IL-10 in mice reveals an immune dysfunction that correlates with the immuno-suppresive effect of IL-10. However, therapies using IL-10 in some bowel inflammatory disorders such as Crohn's disease produce little improvement [180].

During pregnancy, placenta-resident macrophages and T cells, trophoblasts, and decidual cells are the major source of IL-10 [181, 182] [183] [184]. Experiments in abortion-prone CBAx DBA/2 mating mice, whose placenta produces a low level of IL-10 and IL-4, show that the administration of IL-10 prevents fetal loss [149], whereas, the administration of anti-IL10 antibodies to Balb/c pregnant mice causes a transient growth retardation [185].

The fact that IL-10 expression in serum of normal pregnant women as well as in placental explants is incrementally elevated when pregnancy progresses hints at the

physiological function of IL-10 in maintaining gestation [182, 186]. There is an accumulation of evidence that the more common pregnancy complications (e.g., pre-eclampsia, recurrent spontaneous abortion) are associated with low production of IL-10 by immune cells and placenta, while the distribution of IL-10 receptor in the placenta is unchanged [147] [148] [187] [188]. In addition, Orange reported that under *in vitro* stimulated conditions peripheral blood mononulear cells isolated from pre-eclampsia patients could not synthesize the normal levels of IL-10 [189]. This observation is in accordance with a predominantly pro-inflammatory profile of cytokine-expressing T cells isolated in women with recurrent spontaneous abortion or implantation failure after IVF/ET (in-vitro fertilization and embryo transfer) [150]. Remarkably, IL-10 has been shown to inhibit NK activity by inducing trophoblasts and macrophages to express HLA-G, which would suggest a protective role for IL-10 in pregnancy outcome [102].

7.3 Interleukin-6

IL-6 is a multifunctional cytokine that can influence cell-mediated and humoral immune reactions. Activated by bacteria, human macrophages produce IL-6 that in turn induces hepatocytes to synthesize such acute-phase proteins as C-reactive protein, fibrinogen, and mannose-binding protein. These proteins, once binding to bacterial surface, enhance phagocytosis and induce direct lysis of the microorganism. Additionally, in coordination with other Th2 cytokines (IL-4 and IL-5), IL-6 promotes the proliferation and differentiation of B cells into antibody-producing plasma cells that produce specific antibodies against pathogens in a chronic infection [156]. Rincon reported that IL-6 can induce naïve CD4+ T cells to produce IL-4 whereby polarizing them towards Th2 differentiation [190]. The inhibitory effect of IL-6 in the production of

pro-inflammatory cytokines, such as TNFα, IFNγ, and MIP-2, was confirmed with experiments in IL-6-deficient mice stimulated with LPS. It was found that IL-6-/- mice were able to synthesize a significantly higher amount of those cytokines than wild-type mice. Introduction of recombinant IL-6 reverses these pro-inflammatory responses in IL-6 -/- mice [191-194].

The observation that IL-6 is produced in pregnancy by human trophoblasts with a high titer [119] and that it positively regulates the release of human chorionic gonadotropin (hCG) [195] and placental lactogen [196] suggests the role of IL-6 in promoting trophoblast growth. Mouse and human uterine cells have been shown to produce IL-6 constitutively or in response to IL-1 and TNF, respectively [197] [198]. The early expression of mouse IL-6 in decidual tissue suggests a role of IL-6 in remodeling the murine uterine tissue for embryonic implantation [199]. Although IL-6 seems to promote a healthy pregnancy by countering the effect of pro-inflammatory cytokines, IL-6-deficient mice have no apparent defects in reproduction, which may reflect the redundancy of the cytokine network during pregnancy.

8 CYCLOOXYGENASE 2 (COX-2) AND PROSTAGLANDIN E_2 (PGE_2) IN IMMUNITY AND PREGNANCY

8.1 COX-2/PGE_2 metabolism

In mammalian species the two isoforms of the cyclooxygenase (COX-1 and -2) enzyme catalyze the rate-limiting steps in the synthesis of various prostaglandins (PGs) that play a critical role in maintaining homeostasis (as in gastrointestinal protection, kidney-compromised blood supply, and platelet formation), and under certain

circumstances contribute to pathological conditions. While COX-1 is constitutively expressed in most tissues, the expression of COX-2 is inducible and cell-type restricted [200-203]. Arachidonic acid (AA), a lipid metabolite derived from cell-membrane phospholipid as a result of phospholipase A_2 (PLA) metabolism, is the common substrate for the two isoforms [204].

In most tissues, the expression of COX-1 is usually constant. In contrast, the inducible COX-2 becomes highly expressed in response to growth factors, cytokines, and inflammatory factors [205]. Brock et al. suggest that the difference in biological effect generated by the two isoforms is a consequence of shifting PG production. According to their analysis of resting rat macrophages, COX-1 is the primary enzyme implicated in the synthesis of PGI_2, PGD_2, TXA, and HHT in similar amounts, and a smaller quantity of PGE_2. Under LPS stimulation, the *de novo* synthesized COX-2 preferentially catalyzes the conversion of arachidonic acid (AA) into PGE_2 and PGI_2. [206]. In addition, cell-type specific prostaglandin synthases eventually determine the end product of this pathway.

The evidence that COX-2 plays an essential role in reproduction comes from the study of COX-2-deficient mice. While COX-1 -/- females are able to reproduce and only display delayed parturition, the COX-2-/- females show various defects in ovulation, fertilization, implantation, and decidualization. Such reproductive failures can be prevented, to some extent, with prostaglandin supplementation, whereas treatment of COX-1-deficient females with COX-2 specific inhibitor renders them infertile [207] [208-211]. (Figure 6A)

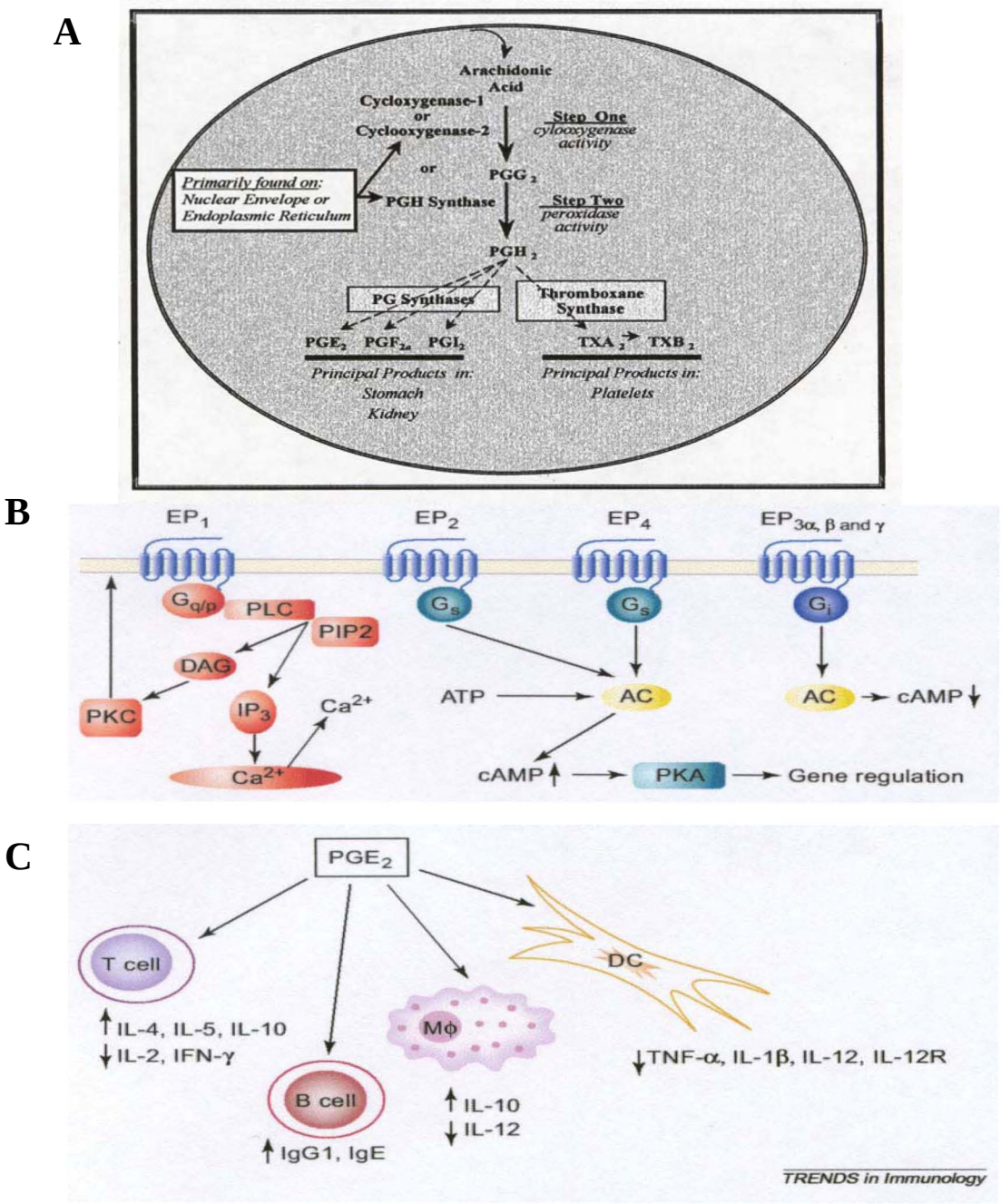


Figure 6. COX-2/PGE_2 metabolism and signaling pathways

A. COX/PGE_2 metabolism

B. Signaling pathways generated by PGE_2-Receptor interaction

C. Immuno-regulation of PGE_2

(Sources: (A) Cryer et al., *Prostaglandins & Other Lipid Mediators*, 56: 341-361, 1998; (B) and (C) Christopher et al., *Trends in Biochemical Sciences*, 28, 2003)

8.2 PGE_2 in the regulation of immunity

PGE_2 plays an important role in mediating the diverse biological activities of homeostasis in many organs, such as cardiovascular, pulmonary, renal, endocrine, reproductive, gastrointestinal, and immune systems. Because of the potent role of PGE_2 in vasodilation and vascular permeability in epithelial cells, the elevated production of PGE_2 correlates with certain symptoms of inflammation (such as edema, pain, and fever). On the other hand, PGE_2 is also believed to possess anti-inflammatory properties that can influence the resolution of inflammation [212].

To date, there is compelling evidence for PGE_2 acting as a powerful immuno-suppressive mediator that can modulate the activities of T cells, B cells, and APCs upon binding to its distinct subtype G-coupled protein receptors (EP1, EP2, EP3, EP4). For instance, in T cells PGE_2 inhibits $CD4^+$ T cell proliferation and induces immature T cell apoptosis. It is also able to inhibit Th1 cell-mediated production of IL-2 and IFNγ while inducing Th2 cells to synthesize IL-4, IL-5, and IL-10. Acting on activated macrophages, PGE_2 has been shown to up-regulate the synthesis of IL-10 while inhibiting the production of pro-inflammatory cytokines [213, 214]. (Figures 6B and 6C)

The paracrine and autocrine effect of PGE_2 also includes a positive feedback in COX-2 synthesis, and the production of PGE_2 is negatively controlled by the prostaglandin catabolizing enzyme PGDH (prostaglandin dehydrogenase) which helps to balance homeostasis [215]. In sum, the output synthesis of PGE_2 results from a complicated network of PGE_2-related metabolic steps (involving PLA_2, COX enzymes, PGE synthase, and PGDH) and various growth-promoting factors and cytokines.

8.3 PGE_2 in pregnancy

In pregnancy, the increased expression of PGE_2 is correlated with the progression of gestation. According to Lim, the activation of PGE_2 via its binding to its subtype receptor EP2 is necessary for blastocyst implantation into the mouse uterus and for decidualization as well, leading to a successful pregnancy [216]. The role of PGE_2 in implantation, decidualization, and labor promotion in other species has also been studied [217-219].

In humans, under the influence of cortisol, fetal membranes including amnion and chorion, and decidual tissues increasingly produce prostaglandins—predominantly PGE_2 —throughout the course of pregnancy. Together with PGF_{2a}, PGE_2 has a positive feed back on cortisol synthesis and participates in the initiation and progression of labor, leading to increased uterine contractility and, finally, delivery at term [220]. While the output of PGs needs to be regulated during gestation, recent experiments involving the administration of PGE_2 to induce labor have shown several interesting and promising clinical aspects. For instance, when used as a vaginal labor inducer for women with post-term pregnancy (gestational age more than a full 41 weeks), PGE_2 has been shown to successfully induce spontaneous labor, compared to the control group which subsequently had a higher incidence of Cesarean deliveries [221].

In addition, PGE_2 has an inhibitory effect on the granulated metrial gland cells, a lineage of NK cells, located at the uterine implantation site altering their morphology and rendering them less cytotoxic [222]. This, together with the aforementioned immune-suppression property of PGE_2 in immune cells, makes PGE_2 a central figure in the regulation of the maternal immune response and fetal tolerance.

9 SIGNIFICANCE, SPECIFIC AIMS, HYPOTHESIS AND APPROACHES

9.1 Significance

Sixteen percent of all recognized pregnancies in the United States end in fetal losses [223] in which spontaneous abortion is a common cause. Past and current studies suggest that a variety of factors help maintain a healthy pregnancy. Among these is a network of cytokines that can influence the innate and adaptive immune response of the mother culminating in fetal tolerance and successful pregnancy.

PSGs have been shown to induce anti-inflammatory cytokines including IL-10, IL-6, and TGFβ_1 in macrophages, and low levels of PSGs in maternal circulation have been shown to correlate with spontaneous abortion and pre-eclampsia, all pointing to a role for PSGs in immune-modulation during pregnancy. The recent discovery of CD9 as the receptor for PSG17 provides us with a powerful tool to verify and characterize with greater specificity the contribution of the PSG family to the maintenance of a successful pregnancy.

This investigation seeks to add to our knowledge on the biological events induced by the ligand-receptor interaction of PSG17 and CD9. Further field studies explore the signaling mechanisms that underlie the immune-regulatory effects of PSGs, potentially taking us one step closer to a molecular understanding of how PSGs influence the outcome of a pregnancy.

9.2 Specific aims

- To characterize the binding of PSG17 to its receptor, CD9, and to determine the key residues in CD9 that are essential for binding to PSG17;

- To examine the involvement of CD9 in cytokine induction upon binding to PSG17 in murine macrophages;
- To explore the signaling pathways involved in the cytokine induction in macrophages in response to PSG17; and
- To clone the receptor for murine PSG19.

9.3 Hypothesis and approaches

We proposed that, upon binding to PSG17, CD9 mediates cytokine induction in murine macrophages and that the COX-2/PGE2 signaling pathway is involved in this up-regulation. To test this hypothesis, we took the following approaches:

First, protein-protein interaction assay was used to examine the direct interaction between the N1-domain of PSG17N and the large extracellular loop of CD9 (CD9EC2), which are produced by CHO cells and bacteria, respectively. In addition, ELISA and FACS were employed to test whether the amino acids SFQ (#173-175), in particular phenylalanine 174, are essential for the binding of PSG17 to CD9-expressing HEK cells.

The role of CD9 in PSG17-mediated cytokine induction was determined by an evaluation by ELISA of the levels of anti-inflammatory cytokines including IL-10, IL-6, and TGFβ_1, and pro-inflammatory cytokine IL-12. The cytokines were measured from the supernatant secreted by murine wild type and CD9-deficient macrophages in response to PSG17. LPS was used as a control for cytokine synthesis of the cell lines.

The effect of the PSG17N-CD9 interaction on PGE_2 and COX-2 synthesis was investigated by measuring PGE_2 in the supernatant of treated cells by ELISA and detecting COX-2 protein expression by immunoblotting. To determine the involvement

of the COX-2/PGE_2 pathway and cAMP-dependent PKA activation in PSG17N-mediated cytokine induction, the levels of secreted cytokines were evaluated in the presence and absence of a COX-2 specific inhibitor and a cAMP-dependent PKA inhibitor.

To identify the receptor for PSG19 plasmids from a RAW 264.7 cDNA expression library were transfected into HEK 293 EBNA cells, and the receptor-expressing cells were selected by panning onto PSG19N coated dishes. Sequencing of the recovered plasmids revealed the identity of CD9 as the receptor. A protein-protein interaction assay was used to confirm the binding of PSG19 to CD9.

PART TWO

PAPERS

Molecular Biology of the Cell
Vol. 14, 5098–5103, December 2003

Direct Binding of the Ligand PSG17 to CD9 Requires a CD9 Site Essential for Sperm-Egg Fusion

Diego A. Ellerman,*† Cam Ha,†‡ Paul Primakoff,*§ Diana G. Myles,‖ and Gabriela S. Dveksler‡

*Department of Cell Biology and Human Anatomy, School of Medicine, University of California Davis, Davis, California 95616; ‡Department of Pathology, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814; and ‖Section of Molecular and Cellular Biology, University of California Davis, Davis, California 95616

Submitted April 18, 2003; Revised July 7, 2003; Accepted August 12, 2003
Monitoring Editor: Mary Beckerle

The function currently attributed to tetraspanins is to organize molecular complexes in the plasma membrane by using multiple *cis*-interactions. Additionally, the tetraspanin CD9 may be a receptor that binds the soluble ligand PSG17, a member of the immunoglobulin superfamily (IgSF)/CEA subfamily. However, previous data are also consistent with the PSG17 receptor being a CD9 *cis*-associated protein. In the current study, CD9 extracellular loop (EC2) specifically bound to PSG17-coated beads, indicating a direct interaction between the two proteins. However, CD9-EC2 did not bind to PSG17-coated beads if the CD9-EC2 had the mutation SFQ (173-175) to AAA, a previously studied mutation in egg CD9 that abolishes sperm-egg fusion. Also, PSG17 bound to 293 T cells transfected with wild-type CD9 but not the mutant CD9. By immunofluorescence, PSG17 bound to wild-type eggs but not to CD9 null eggs. The presence of ~2 μM recombinant PSG17 produced a significant and reversible inhibition (60–80%) of sperm-egg fusion. Thus, we conclude that CD9 is a receptor for PSG17 and when the PSG17 binding site is mutated or occupied, sperm-egg fusion is impaired. These findings suggest that egg CD9 may function in gamete fusion by binding to a sperm IgSF/CEA subfamily member and such proteins have previously been identified on sperm.

INTRODUCTION

Mammalian fertilization involves a series of complex cellular and biochemical processes, culminating in sperm-egg fusion and subsequent formation of an embryo. Despite its biological importance, fusion between the plasma membranes of the sperm and the oocyte is not well understood. Although there is general agreement on the concept that gamete fusion may involve the interaction of multiple complementary molecules on the sperm and the oocyte, only a few potentially relevant proteins have been identified.

A protein found on the mammalian egg surface that is known to be required for gamete fusion is CD9. CD9 belongs to the tetraspanin family of proteins, which are integral membrane proteins with four transmembrane domains and two extracellular domains (one short, one long) (Maecker *et al*., 1997). In mammals there are >30 tetraspanin family members, implicated in a variety of cellular and physiological processes, such as cell motility, cell aggregation, signaling, and cell fusion (Boucheix and Rubinstein, 2001; Hemler, 2001). Tetraspanins are believed to act as "molecular facilitators," grouping specific cell-surface proteins and thus increasing the formation and stability of functional protein complexes (Maecker *et al*., 1997). CD9 associates with a great variety of membrane proteins, such as membrane-anchored growth factors, integrins, members of the immunoglobulin superfamily (IgSF), and other tetraspanins (Boucheix and Rubinstein, 2001).

Conclusive evidence for a role of CD9 in gamete fusion was the finding that CD9 knockout females are infertile due to the inability of their oocytes to fuse with sperm (Kaji *et al*., 2000; Le Naour *et al*., 2000; Miyado *et al*., 2000), but how CD9 acts in fusion remains unknown. It has been suggested that CD9 acts in sperm-egg fusion via an association with a β1 integrin on the egg that binds to ADAM proteins on the sperm, specifically ADAM 2 (fertilin β) and ADAM 3 (cyritestin) (Chen *et al*., 1999; Takahashi *et al*., 2001). However, experiments using gametes deficient for either the sperm ADAMs or the egg integrins, question the validity of this model. Sperm lacking fertilin β, cyritestin, or both proteins are still able to fuse with eggs (Cho *et al*., 1998; Shamsadin *et al*., 1999; Nishimura *et al*., 2001). Furthermore, it was recently shown that oocytes lacking all β1 integrins are fully functional in fusing with sperm in vitro and in vivo. Also function-blocking antibodies against other egg integrins (β3 and αv), when incubated with eggs lacking β1 integrins, do not inhibit sperm-egg fusion (He *et al*., 2003). These results indicate that none of the integrins known to be present on the oocyte is essential for sperm-egg fusion and egg integrins do not have redundant functions in the fusion process.

Because the proposed mechanism for CD9 function in sperm-egg fusion, acting through integrins, does not agree with these current results, an alternative model is required. Recently, PSG17, a member of the pregnancy-specific glycoprotein (PSG) family, has been suggested to be a CD9-ligand (Waterhouse *et al*., 2002). Pregnancy-specific glycoproteins

Article published online ahead of print. Mol. Biol. Cell 10.1091/mbc.E03–04–0244. Article and publication date are available at www.molbiolcell.org/cgi/doi/10.1091/mbc.E03–04–0244.

† These authors contributed equally to this work.

§ Corresponding author. E-mail address: pdprimakoff@ucdavis.edu.

belong to the carcinoembryonic antigen (CEA) subfamily of the immunoglobulin superfamily (IgSF) (Beauchemin *et al*., 1999). PSGs are a group of proteins synthesized by the placenta and secreted into the maternal circulation. Several PSGs are known to stimulate secretion of cytokines by macrophages (Wessells *et al*., 2000; Snyder *et al*., 2001), and thus PSGs may contribute to preventing fetal rejection by the mother by activating the maternal innate immune system (Sacks *et al*., 1999). PSG17 binds to macrophages with high affinity and the binding is mediated by CD9 (Waterhouse *et al*., 2002). However, it is not known whether CD9 is the receptor itself or whether it functions as a coreceptor.

We report here that PSG17 binds directly to CD9 and that CD9 amino acid residue F 174 is essential for this interaction. As a CD9-ligand molecule, PSG17 interactions may give insights into the molecular mechanism underlying the role of CD9 in sperm-egg fusion. We found that PSG17 binds to eggs and inhibits gamete fusion, suggesting that CD9 may function in sperm-egg fusion by interacting with an IgSF protein, possibly a CEA subfamily protein. Thus, these results provide evidence for the potential involvement of an IgSF member in sperm-egg fusion and give rise to new ideas of how CD9 acts in this process.

MATERIALS AND METHODS

Cell Culture

Human embryonic kidney (HEK) 293T cells (Edge Biosystems, Gaithersburg, MD) were cultured in DMEM, 10% fetal bovine serum. BeWo cells (American Type Culture Collection, Manassas, VA) were cultured in F12K medium with 1.5 g/l sodium bicarbonate and 10% fetal bovine serum.

Plasmids

The construction of pGEX-CD9EC2, pCD9-eGFP, and pCD9-F174A-eGFP has been described previously (Zhu *et al*., 2002). For the expression of glutathione *S*-transferase (GST)-CD9EC2-SFQ to AAA, the mutated template (Zhu *et al*., 2002) was amplified by polymerase chain reaction and subcloned into *Eco*RI and *Bam*HI restriction sites of pGEX-3× (Amersham Biosciences, Piscataway, NJ). pCD9-SFQ to AAA (173-175)-eGFP was generated after digestion of the cDNA in pBluescript KS (Zhu *et al*., 2002) with *Sal*I and *Sac*I and subcloned into the same sites in the pIRES2-eGFP vector (BD Biosciences Clontech, Palo Alto, CA).

Pull-Down Assays

The GST-mouse CD9 extracellular loop 2 (EC2) fusion protein was generated as described previously (Zhu *et al*., 2002). Recombinant PSG17N-Myc-His includes the N-terminal immunoglobulin domain of PSG17 and binds with high affinity to CD9-expressing cells (Waterhouse *et al*., 2002). Recombinant PSG17N-Myc-His purified as described in Waterhouse *et al.* (2002) was tested for its ability to bind to GST-CD9EC2 with the pull-down polyHis protein: protein interaction kit (Pierce Chemical, Rockford, IL) following the manufacturer's protocol. Briefly, 100 μg of purified PSG17N-Myc-His or two control proteins, recombinant green fluorescent protein (GFP)-His (Upstate Biotechnology, Lake Placid, NY) or CEACAM1a[1-4]-His (Zelus *et al*., 1998; Beauchemin *et al*., 1999), were incubated with the immobilized cobalt chelate gel overnight at 4°C. After five washes, 100 μg of GST-CD9EC2 (prey) or the same construct carrying the SFQ (173-175) to AAA mutation were added to the gel and incubated overnight with gentle rocking at 4°C. After seven washes, the proteins were eluted with 150 μl of 290 mM imidazole elution buffer. Twenty-five microliters of the eluted material was loaded on a 4–20% NuPAGE gel (Invitrogen, Carlsbad, CA), and the proteins were detected by immunoblotting with an anti-CD9 mAb, KMC8 (BD Pharmingen, Palo Alto, CA), or an anti-GST monoclonal antibody (mAb) (Santa Cruz Biotechnology, Santa Cruz, CA) followed by horseradish peroxidase-conjugated goat anti-rat antibody or goat anti-mouse antibody and the Super Signal chemiluminescent detection system (Pierce Chemical).

Detection of PSG17N-Myc-His Binding to Transfected 293T Cells by Enzyme-linked Immunosorbent Assay (ELISA)

The assays were performed as described previously (Waterhouse *et al*., 2002). Briefly, HEK 293T cells were seeded in poly-L-lysine–coated 96-well plates and were transiently transfected with plasmid DNA by using LipofectAMINE 2000 (Invitrogen). At 48 h posttransfection, the cells were washed with binding buffer containing 0.01% sodium azide after which PSG17N-Myc-His (10 μg/ml) or no ligand was added to each well. After 1-h incubation, the cells were washed five times and binding of PSG17N-Myc-His was detected with horseradish peroxidase-conjugated anti-Myc mAb (Invitrogen) followed by trimethylbenzyl-peroxidase substrate (KPL) and 2 N H_2SO_4. The color change was quantitated at 450 nm in an ELISA reader.

Flow Cytometry

For detection of PSG17N-Myc-His binding to 293T cells transfected with pCD9-eGFP, pCD9-F174A-eGFP, and pCD9-SFQ to AAA (173-175)-eGFP, the cells were sequentially incubated with 10 μg/ml PSG17N-Myc-His, anti-Myc mAb, and phycoerythrine (PE)-labeled rat anti-mouse IgG1 (BD Pharmingen). Expression of wild-type CD9, and the mutated forms of CD9 on the surface of the transfected cells, was confirmed by staining with biotin-labeled anti-CD9 mAb, KMC8, followed by Cy-Chrome-labeled streptavidin (BD Pharmingen).

Gamete Isolation

Mature, cumulus-free oocytes were collected from superovulated 6–8-wk-old ICR female mice as described previously (Yuan *et al*., 1997). To loosen the zona pellucida (ZP) the oocytes were treated with 30 μg/ml chymotrypsin (Sigma-Aldrich, St. Louis, MO) for 3 min at 37°C and 5% CO_2 in medium M199 (Invitrogen) containing 3.5 mM sodium pyruvate, 1000 IU of penicillin-streptomycin, and 0.3% bovine serum albumin (BSA) (Sigma-Aldrich) (M199*). The zonae pellucidae were then removed mechanically using a narrow bore pipette. The oocytes were washed through three 100-μl drops of fresh M199* and then incubated in M199* at 37°C and 5% CO_2 for 3 h before use. Sperm were collected from the cauda epididymis and vas deferens of 10–12-wk-old ICR males. Sperm were allowed to disperse in a 500-μl drop of M199* containing 3% BSA and then diluted 1:10 in 500 μl of M199* + 3% BSA and capacitated for 2 h at 37°C and 5% CO_2.

Egg Immunofluorescence

Wild-type or CD9-null zona-free oocytes were obtained as described above and incubated in 50 μg/ml PSG17N-Myc-His (~26 kDa) or XylE-His (~35 kDa) in M199* for 30 min at 37°C in a 5% CO_2 incubator. After three washes in M199*, the cells were incubated in anti-Myc antibody (Invitrogen) (10 μg/ml in phosphate-buffered saline [PBS]-0.4% BSA) for 1 h at room temperature. Oocytes were washed through three drops of PBS-0.4% BSA and then exposed to Oregon green-conjugated anti-mouse IgG secondary antibody (Molecular Probes, Eugene, OR) diluted 1:100 in PBS-0.4% BSA for 1 h at room temperature. After washing, oocytes were mounted on glass slides and visualized using a Zeiss Axiophot microscope.

In Vitro Fertilization Assay

Zona-free eggs were loaded with 4′,6-diamidino-2-phenylindole dihydrochloride (Polysciences, Warrington, PA) at 10 μg/ml for 15 min at 37°C, 5% CO_2. After washing out excess dye, the eggs were incubated for 30 min at 37°C and 5% CO_2 in M199* alone (control) or M199* containing 50 μg/ml PSG17N-Myc-His (~2 μM). Oocytes were inseminated in droplets containing recombinant protein for the first set of in vitro fertilization experiments, or were washed in M199* alone, by using three successive 7-min incubations in 100-μl M199* drops at 37°C, 5% CO_2 before insemination, for the in vitro fertilization experiments testing the reversibility of the inhibition. Sperm were added at a final concentration of 1–3 × 10^5 sperm/ml, and gametes were coincubated for 40 min at 37°C, 5% CO_2. The oocytes were then washed to release loosely bound sperm, fixed in 4% paraformaldehyde in PBS for 10 min, and mounted onto microscope slides. Sperm-egg fusion was scored by the fluorescent labeling of sperm nuclei by 4′,6-diamidino-2-phenylindole transferred from the preloaded eggs. Two different parameters of fusion were calculated: fertilization rate is the percentage of oocytes with at least one fused sperm, and fertilization index is the mean number of fused sperm per egg.

Sperm Motility Assay

Capacitated sperm were incubated for 30 min at 37°C, 5% CO_2 in M199* alone (control) or containing 50 μg/ml PSG17N-Myc-His. Samples were placed on warm glass slides and analyzed using a light microscope. Cells showing progressive motility were counted as "motile," whereas cells with nonprogressive motility or no motility were considered as "immotile."

RESULTS

Direct Binding of PSG17 to CD9

Previous results demonstrated that PSG17 binds specifically to CD9-expressing cells (Waterhouse *et al*., 2002), but it has been uncertain whether PSG17 binds to CD9 itself or to a CD9-associated protein. To test whether PSG17 binds di-

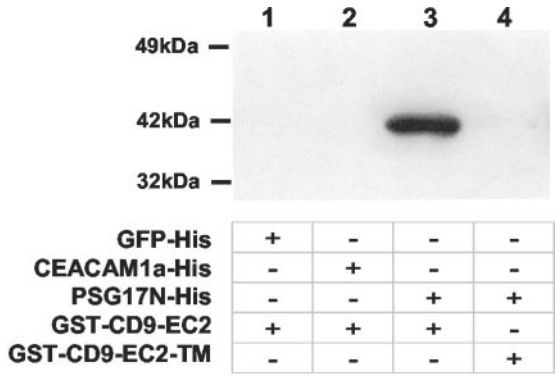


Figure 1. PSG17N binds to the extracellular loop 2 of murine CD9. Cobalt chelate beads were incubated with GFP-His (lane 1), CEACAM1a[1-4]-His (lane 2) or PSG17N-Myc-His (lanes 3 and 4) used as baits. The beads were then incubated with GST-CD9EC2 (lanes 1–3) or the same construct carrying the triple mutation SFQ (173-175) to AAA (GST-CD9EC2-TM) (lane 4). After several washes, the proteins were eluted with a buffer containing 290 mM imidazole, separated on a 4–20% NuPAGE gel, and detected by Western blot by using an anti-CD9 antibody.

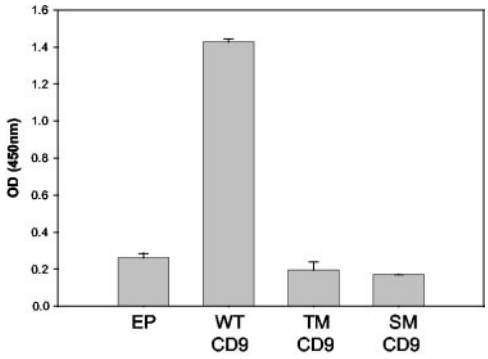


Figure 2. Binding of PSG17N-Myc-His to CD9-transfected 293T cells. HEK 293T cells were transfected with empty plasmid (EP), pCD9-eGFP (wild-type CD9:WT-CD9), pCD9-F174A-eGFP (single mutant CD9: SM-CD9), or pCD9-SFQ to AAA (173-175)-eGFP (triple mutant CD9: TM-CD9). Forty-eight hours posttransfection, the cells were incubated with 10 μg/ml PSG17N-Myc-His and bound PSG17N-Myc-His was detected after treatment with HRP-conjugated anti-Myc mAb and TMB/peroxidase substrate. The data are expressed as mean absorbance $\pm$ SE. Each data point represents four identical wells and the experiment was repeated two independent times with similar results.

rectly to CD9, pull-down assays were performed. The recombinant N-terminal Ig domain of PSG17 containing both 6 $\times$ His and Myc epitope tags was bound through its His tag to cobalt beads. Two control proteins were used, eGFP-6 $\times$ His and CEACAM1a[1-4]-6 $\times$ His. CEACAM1a is closely related to PSG17; it is also an IgSF member and, like PSG17, belongs to the CEA subfamily. Recombinant CEACAM1a[1-4] has also been shown to have biological activity (Zelus *et al*., 1998). The beads with bound protein were incubated with a fusion protein, including GST fused to the CD9 long extracellular loop (EC2) (GST-CD9 EC2). After extensive washing, the presence of CD9 on the beads was detected by Western blot by using an anti-CD9 antibody. GST-CD9 EC2 bound to PSG17-coated beads but not to the GFP-coated beads or to the CEACAM1a[1-4]-coated beads (Figure 1, lanes 1–3), showing that PSG17 binds directly to CD9-EC2.

PSG17 Binds to a Specific Site on CD9

It has been previously shown that amino acid residues 173–175 in CD9 EC2 are critical for CD9 function in sperm-egg fusion. When CD9 EC2 residue F174 is replaced by A, CD9 activity in gamete fusion is greatly reduced; when the three residues (173–175) are altered to AAA, the mutant CD9 has no activity in fusion (Zhu *et al*., 2002). To ask whether this region of CD9 is also relevant for the binding of PSG17, a GST-CD9 EC2 construct with the triple mutation SFQ to AAA was expressed and used in the pull-down assay. In contrast to the wild-type construct, the mutated CD9 EC2 was not pulled down by PSG17-coupled beads (Figure 1, lane 4), suggesting that amino acid residues 173–175 are directly involved in binding to PSG17.

We also evaluated whether mutations in CD9 would affect PSG17 binding when CD9 was expressed in a cell membrane. We compared by ELISA the binding of PSG17 to HEK 293T cells that had been transfected with either wild-type CD9 or mutant CD9. Two CD9 mutations were used, the triple mutant, residues 173–175 SFQ to AAA, mentioned above, or the single mutant, residue F174 to A. Compared with control cells transfected with empty plasmid, wild-type CD9 transfected cells bound about five times higher levels of PSG17 (Figure 2). Cells transfected with CD9 with the triple mutation (SFQ 173–175 to AAA) or CD9 with the single mutation (F 174 to A) bound PSG17 at the same level as the control (empty plasmid) transfected cells. Cells transfected with any of the three constructs showed similar surface staining by immunofluorescence by using the anti-CD9 KMC8 antibody (our unpublished data).

Similar results were obtained using fluorescence-activated cell sorting (FACS) analysis of PSG17 binding to CD9-transfected 293T cells. 293T cells were transfected with plasmids coding for wild-type or mutant CD9 fused to enhanced green fluorescent protein (eGFP). Transfected cells were incubated with PSG17-N-Myc-His and stained with an anti-Myc antibody followed by a secondary antibody coupled to PE. Expression levels of the CD9-eGFP fusion proteins were evaluated by the fluorescence of eGFP and PSG17 binding was quantified by PE fluorescence. Results indicated that eGFP and PE-labeled double positive cells constituted 37% of the forward versus side scatter-gated pCD9-eGFP transfected cells (Figure 3). In contrast, only 0.4% of the gated cells were eGFP and PE double positive when cells were transfected with pCD9-F174A-eGFP. Results similar to those with the F174A mutant were obtained upon transfection of cells with CD9-SFQ to AAA (173–175)-eGFP encoding plasmid (our unpublished data). Together, these results show that PSG17 binds to CD9 and that F174 is critical for the interaction with PSG17.

Binding of PSG-17 to Eggs and Inhibition of Sperm-Egg Fusion

Considering the essential role CD9 has in sperm-egg fusion, and in light of the evidence above supporting a direct interaction between PSG-17 and CD9, we tested whether PSG17 binds to eggs and affects gamete fusion. The presence of CD9 on the eggs has been demonstrated (Chen *et al*., 1999). However, CD9 is known to have multiple *cis*-partners and the complement of CD9 *cis*-partners may be cell type specific, so the accessibility of the PSG17 binding site on the egg could be different from that on macrophages or HEK 293 cells. To

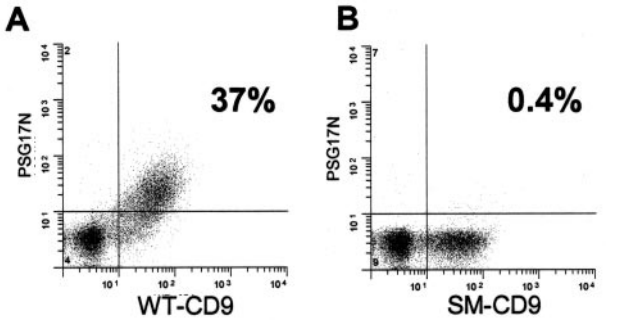


Figure 3. FACS analysis of PSG17N-Myc-His binding to HEK 293T wild-type and mutated CD9-transfected cells. HEK 293T cells were transfected with pCD9-eGFP (wild-type CD9: WT-CD9) (A) or pCD9-F174A-eGFP (single mutant CD9: SM-CD9) (B), after which they were sequentially incubated with 10 μg/ml PSG17N-Myc-His, anti-Myc mAb, and PE-labeled rat anti-mouse IgG1.

explore whether PSG17 is able to bind to eggs, zona-free oocytes were incubated with PSG17N-Myc-His and then examined by indirect immunofluorescence by using an anti-Myc antibody. Oocytes incubated in the presence of a control His-tagged protein (XylE) showed no fluorescence (Figure 4, A and B), whereas oocytes incubated with PSG17 displayed a bright staining (Figure 4, C and D). To confirm that PSG17 binding is CD9-dependent, we examined whether CD9 null eggs were able to bind PSG17 and found no staining in CD9 KO eggs (Figure 4, E and F).

Next, we asked whether binding of PSG17 to CD9 would affect the ability of eggs to fuse with sperm. Zona-free eggs were preincubated with PSG17, and then inseminated with capacitated sperm. After a coincubation period of 40 min, the percentage of fertilized eggs and the mean number of fused sperm per egg were determined. Oocytes preincubated with PSG17 had a significantly lower fertilization rate (58% inhibition) and fertilization index (83% inhibition) than control oocytes (Figure 5). Sperm motility was unaffected by the presence of PSG17 (61% motility with PSG17, 58% motility in the absence of PSG17). Immunofluorescence experiments showed that sperm did not bind PSG17 during coincubation with the eggs, suggesting that the oocyte is the targeted gamete. To study whether the oocyte was blocked in a reversible or irreversible manner, oocytes were incubated with PSG17, washed, and then inseminated with capacitated sperm. Eggs, exposed to PSG17 and then washed, had a fertilization rate and a fertilization index significantly higher than those of the nonwashed oocytes, and not significantly different from the values obtained for control oocytes (Figure 6). These results indicate that the inhibitory effect could be reversed by PSG17 removal and argue against a toxic effect of the protein preparation on the oocytes.

DISCUSSION

Tetraspanins are known to associate with a variety of different molecules, such as membrane-anchored growth factors, integrins, other tetraspanins, and members of the IgSF. A key feature of these reported molecular interactions is that all of them occur in *cis*, i.e., between the tetraspanin and other transmembrane proteins anchored in the same lipid bilayer (Maecker *et al*., 1997; Boucheix and Rubinstein, 2001). One *trans*-interaction has been reported for a tetraspanin, the association between CD81 and the Hepatitis C virus envelope glycoprotein E2. There is evidence indicating that the tetraspanin CD81 interacts in vitro with the virus protein (Flint *et al*., 1999), and key amino acids in CD81 for this

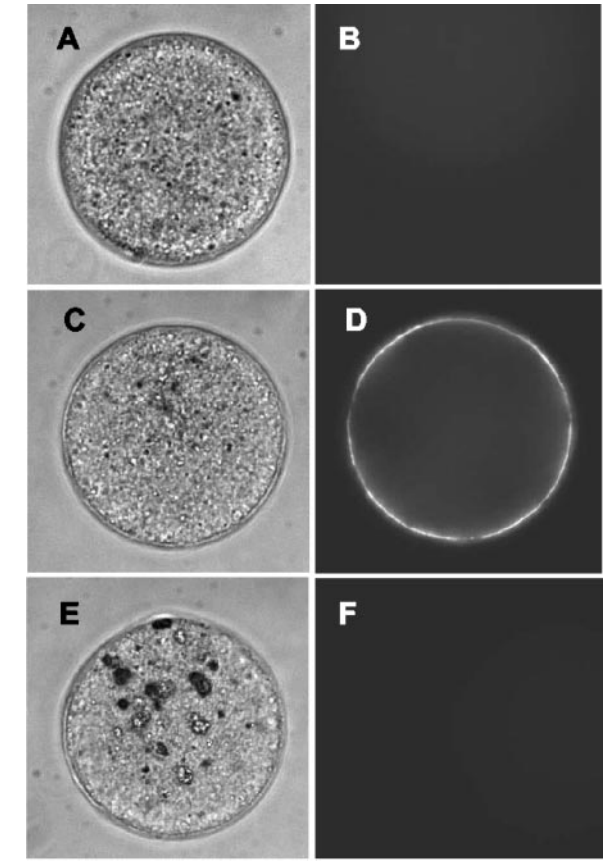


Figure 4. Binding of PSG17N-Myc-His to the oocyte. Zona-free eggs from wild-type (A–D) or CD9 knockout animals (E and F) were incubated in 50 μg/ml XylE (a His-tagged control protein) (A and B) or PSG17N-Myc-His (C–F) for 30 min, and stained by indirect immunofluorescence by using an anti-Myc antibody, followed by Oregon Green-conjugated secondary antibody.

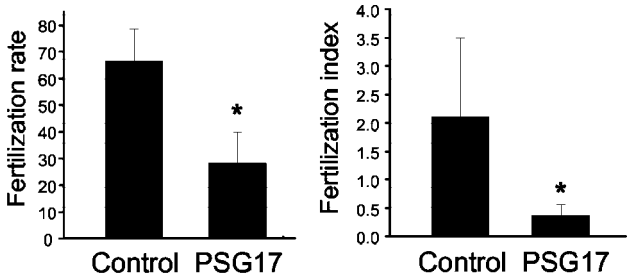


Figure 5. Inhibition of sperm-egg fusion in the presence of PSG17N-Myc-His. Zona-free eggs were incubated for 30 min in medium (Control) or in medium containing PSG17N-Myc-His (50 μg/ml), and then inseminated with capacitated sperm. After coincubating the gametes for 40 min, oocytes were washed and the fertilization rate (number of fertilized eggs/total number of eggs) and the fertilization index (mean number of sperm fused per egg) were scored. Data represents the mean value $\pm$ SE from four independent experiments. *$p < 0.05$ compared with control.

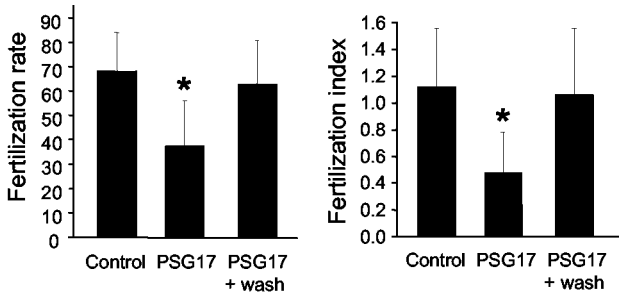


Figure 6. Reversibility of sperm-egg fusion inhibition by PSG17N-Myc-His. Zona-free eggs were incubated for 30 min in medium containing PSG17N-Myc-His (50 μg/ml), washed through 3 drops of medium, and then inseminated with capacitated sperm (PSG17 + wash). Control groups consisted of oocytes incubated in medium (Control), or in the presence of PSG17N-Myc-His without its subsequent removal by washing (PSG17). After coincubating the gametes for 40 min, oocytes were washed and the fertilization rate and the fertilization index were scored. Data represents the mean value ± SE from three independent experiments. *$p < 0.05$ compared with control and to PSG17 + wash.

interaction have been identified within the EC2 loop (Flint *et al*., 1999; Drummer *et al*., 2002). Nevertheless, the interaction between CD81 and E2 does not seem to mediate virus entry (Petracca *et al*., 2000; Takikawa *et al*., 2000) and there are other molecules proposed to function as Hepatitis C virus receptors (Germi *et al*., 2002; Scarselli *et al*., 2002), so the biological significance of the CD81–E2 interaction is uncertain.

Previous work implicated CD9 in the binding of an external ligand, PSG17, to macrophages (Waterhouse *et al*., 2002). One of the aims of the present study was to determine whether CD9 mediates the binding of PSG17 directly or, on the contrary, whether it functions in an indirect way, enhancing the binding of PSG17 to a CD9-associated protein. Results of pull-down experiments, carried out with the purified recombinant proteins, indicate PSG17 binds directly to the EC2 loop of CD9, thus confirming that CD9 is the actual receptor for PSG17. Because PSG17 is the first molecule known to be a biological ligand for a tetraspanin, the association between PSG17 and CD9 represents an unexpected, novel interaction in which the tetraspanin is a receptor.

The EC2 loop of tetraspanins, particularly the stretch of residues preceding the most C-terminal cysteine, is a region in which tetraspanin-associated proteins bind (Stipp *et al*., 2003). This region in CD9 is also the location of CD9 mutations that affect gamete fusion. A striking feature of our data is that the same CD9 mutations that affect CD9 activity in gamete fusion abolish CD9-PSG17 binding. In previous work, we found that CD9 with F (174) mutated to A has greatly reduced activity in gamete fusion and CD9 with SFQ (173–175) mutated to AAA is no longer active in gamete fusion (Zhu *et al*., 2002). In our current results, use of the F to A or SFQ to AAA mutants indicate that these residues are also essential for CD9 binding to PSG17. A possible trivial explanation for these results could be that misfolding of CD9 is produced by the amino acid changes. However, substantial data indicate that the mutated protein (SFQ to AAA) has correct folding (Zhu *et al*., 2002), and therefore it is likely that CD9 residues SFQ 173-175 represent part of a PSG17 binding site.

Alternative explanations exist for how soluble PSG17 might inhibit gamete fusion. Because PSG17 binding initiates signal transduction in macrophages, it is possible that PSG17 binding to egg CD9 produces a similar effect that might trigger egg activation. However, oocytes exposed to PSG17 and then washed, showed a fertilization rate and index similar to control oocytes, indicating that the inhibition is reversible, and therefore arguing against an induction of egg activation by PSG17. Another possibility is that the binding of PSG17 inhibits gamete fusion by displacing a CD9 *cis*-partner from its normal association with CD9. The two most abundant and tightly associated CD9 *cis*-partners in tissue culture cells are both members of the EWI subfamily of the IgSF, EWI-F, and EWI-2 (Charrin *et al*., 2001; Stipp *et al*., 2001a,b). Given the fact that CD9 associates in *cis* with these IgSF members and that PSG17 is also an IgSF member, one can speculate that PSG17 disrupts the *cis*-association between CD9 and egg EWI-F or EWI-2. However, the EWI subfamily and the CEA subfamily (of which PSG17 is a member) have relatively little sequence relationship aside from both possessing Ig domains, making this hypothesis less attractive. Further exploration of this possibility will require the identification of CD9-associated proteins in the oocyte and their binding site(s) in CD9.

Another explanation of our findings is that egg CD9 may bind in *trans* to a PSG17-related ligand present on the sperm surface. When the CD9 SFQ site is mutated or already occupied by soluble PSG17, the sperm surface ligand cannot bind to egg CD9 and this essential step in gamete fusion is blocked. Relevant to this interpretation are two separate findings in our previous article (Zhu *et al*., 2002). One was that soluble CD9-EC2 when preincubated with eggs, inhibits gamete fusion, indicating that CD9-EC2 interacts with another egg surface protein. A second finding was that the SFQ sequence in CD9 is required for fusion, but the issue of SFQ's acting in *trans* or *cis* was not addressed. If CD9 has a *trans*-interaction, we would propose it is in addition to the *cis*-interaction indicated by the previous finding.

Although CD9-EC2 inhibits gamete fusion when preincubated with eggs, it has no effect on fusion when preincubated with sperm. We previously suggested this could mean that egg CD9 does not bind to sperm (Zhu *et al*., 2002), but other explanations of this result are possible. For instance, a sperm *trans*-ligand for CD9 may be inactive or inaccessible until after initial steps in sperm-egg adhesion occur and CD9 is positioned to interact with the *trans*-ligand. Once these adhesion steps occur, the *trans*-ligand becomes activated or accessible to bind the egg surface CD9 in preference to the soluble CD9-EC2.

A sperm *trans*-ligand for CD9 might be a membrane-associated form of PSG17 or a related CEA member. A CEA protein has been identified on the sperm surface and named "sperad." Sperad, initially called AH-20 (Primakoff and Myles, 1983), has been described in guinea pig sperm (Quill and Garbers, 1996). Relevant to sperad's biological function, monoclonal antibodies G3 and G11 stained the equatorial region of acrosome-reacted guinea pig sperm and were able to completely inhibit the fusion of guinea pig sperm with hamster oocytes (Allen and Green, 1995). Sperad was recently reported to be the protein recognized by antibodies G3 and G11 (Ilayperuma, 2002, 2003). Thus, current findings include 1) CD9 is required for sperm-egg fusion; 2) CD9 binds PSG17, a member of the CEA subfamily, and PSG17 inhibits sperm-egg fusion; and 3) there is a CEA protein on sperm that has been implicated in sperm-egg fusion. Together, these results support the idea that CD9 may function in gamete fusion by binding to a sperm CEA protein.

During recent years models for gamete fusion have focused on an adhesion role of a sperm ADAM(s) binding to an egg integrin(s) and CD9 was implicated as facilitator of this interaction (Takahashi *et al*., 2001; Evans, 2002). Recent data have raised doubts about the participation of ADAMs and integrins in sperm-egg fusion (Primakoff and Myles, 2002; He *et al*., 2003), although CD9 is clearly required. Our current findings suggest the participation in gamete fusion of IgSF proteins that bind to CD9. In this study we found that CD9 is a receptor for an IgSF/CEA subfamily ligand, PSG17, which binds to a CD9 site, including residues SFQ 173-175, known to be an active site for gamete fusion. Further work should reveal whether during gamete fusion the egg SFQ site binds an IgSF/CEA ligand on the sperm surface and/or is essential for CD9 *cis*-interactions in the egg plasma membrane.

ACKNOWLEDGMENTS

We are grateful to K. Wolcott for technical assistance in the FACS analysis and to Dr. Kathryn V. Holmes (Department of Microbiology, University of Colorado Health Sciences Center) for supplying the recombinant CEACAM1a[1-4]-His protein. This work was supported by National Institutes of Health grants HD35832 (to G.D.) and HD16850 (to D.G.M.).

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Binding of Pregnancy Specific Glycoprotein 17 to CD9 on Macrophages Induces Secretion of IL-10, IL-6, PGE_2, and TGF-β1[1]

Cam Ha[*], Roseann Waterhouse[*], Jennifer Wessells[†], Julie A. Wu [*] and Gabriela S. Dveksler[2*]

[*] Department of Pathology, Uniformed Services University of the Health Sciences, Bethesda, MD 20814-4799; and †Laboratory of Protein Dynamics and Signaling, National Cancer Institute-Frederick, Frederick, MD 21702-1201

[1] This work was supported by Grant HD35832 from the National Institutes of Health

[2] Corresponding author: Gabriela S. Dveksler, Department of Pathology, USUHS, 4301 Jones Bridge Rd., Bethesda, MD 20814, USA. E-mail address: gdveksler@usuhs.mil. Phone: 301-295-3332. Fax: 301-295-1640

[3] Key words: Reproductive immunology/ cytokines/ macrophages

[4] Running title: Macrophage CD9-pregnancy specific glycoprotein interaction

[5]Abbreviations used in this paper: BMDM, J2 transformed bone marrow derived macrophage; CD9EC2, CD9 extracellular loop 2; CD9WT, CD9 wild type; CD9KO, CD9 knockout; COX-2, cyclooxygenase-2; cAMP-dependent PKA, cAMP-dependent protein kinase A; PGE_2, prostaglandin E_2 ; PSG, pregnancy specific glycoprotein

Abstract

Pregnancy specific glycoproteins (PSGs) are a family of secreted proteins produced by the placenta. In humans, the serum concentration of these glycoproteins exceeds that of other pregnancy-related proteins, reaching up to 200-400 μg/ml at term. In species with hemochorial placentation, PSGs are believed to have a critical role in the success of pregnancy. We previously reported that treatment of human monocytes with PSGs induced IL-10, IL-6, and TGF-β_1 secretion. Although the receptor for human PSGs remains unknown, we identified CD9 as the receptor for both murine PSG17 and PSG19. To determine whether human and murine PSG family members have similar functions and whether CD9 mediates the response to PSG17, we treated macrophages from wild type and CD9-deficient mice with recombinant murine PSG17N, which consists of the N_1-domain of this glycoprotein. Our data reveal that CD9 expression is required for PSG17N-induced secretion of IL-10, IL-6, PGE_2, and TGF-β_1 in macrophages. The ability of PSG17N to induce IL-10 and IL-6 mRNA was significantly reduced in the presence of cycloheximide, and secretion of these cytokines required induction of cyclooxygenase-2. Further characterization of the response to PSG17 indicated that cAMP-dependent protein kinase A is involved in the up-regulation of IL-10 and IL-6. The induction of anti-inflammatory cytokines by various PSGs supports the hypothesis that these placenta-secreted glycoproteins have an essential role in the regulation of the maternal immune response during pregnancy.

Introduction

Pregnancy specific glycoproteins (PSGs) are a family of highly homologous proteins secreted by the placenta. They were originally isolated from the circulation of pregnant women [1]. PSGs are detected in maternal blood as early as seven days post implantation. The serum levels of these proteins reach up to 200-400 μg/ml at term, far exceeding the concentration of human chorionic gonadotropin and alpha fetoprotein [2]. Abnormally low levels of PSGs are associated with several serious complications of pregnancy including fetal hypoxia, fetal growth retardation, pre-eclampsia and spontaneous abortion [3,4,5,6]. PSG homologues have been identified in mice, rats, and primates [7,8,9]. Treatment of monkeys and mice with anti-PSG antibodies resulted in spontaneous abortion [10] [11]. Collectively, the high concentration of PSGs in maternal serum, their linkage to fetal pathologies, and experimentally induced abortions provide strong evidence that PSGs play a key role in pregnancy.

We have previously shown that treatment of human monocytes with recombinant human PSG1, PSG6, and PSG11 induced the production of anti-inflammatory cytokines [12]. Interestingly, activated monocytes from women with recurrent spontaneous abortion display a decrease in PSG11-induced expression of IL-10 [13]. Together these findings suggest that human PSGs are key immune regulatory factors.

There are 15 murine PSG genes (*psg 16-30*) localized to chromosome 7 in a region syntenic to human chromosome 19 [8]. Murine PSGs are similar to human PSGs in that they contain several Ig-like domains and are heavily glycosylated. We have demonstrated for several murine and human PSGs that the N-terminal Ig-variable-like domain is sufficient to elicit biological activity [12] [14].

Previously we reported that the receptor for PSG17 in macrophages is the tetraspanin family member CD9 [15] and that PSG17N binds to the large extracellular loop of CD9 [16]. CD9 is a 24-27 kDa transmembrane protein that is expressed abundantly in various tissues- hemapoietic as well as non-hemapoietic [17] [18]. Like other members of the family, CD9 has been suggested to act as a linker for its associated molecules and therefore in the formation of the tetraspanin web, a specific stimulation can trigger or prevent specific signaling pathways [19] [20]. CD9-associated molecules have been found to include $\alpha_{(3-4)}\beta_1$ integrins, PKC, PI4K, tetraspanin members, and EWI family [21, 22, 23]. However, the identity of the receptor for human PSGs remains unknown. In this study, we investigated the induction of cytokine secretion by murine PSG17N in the murine macrophage cell line RAW 264.7 and thioglycollate-induced peritoneal macrophages from Th1- and Th-2 prone mice. In addition, we examined the response to PSG17N in bone marrow-derived macrophages obtained from wild type and CD9-deficient mice. Furthermore, we took the first steps to elucidate the signaling mechanisms responsible for the CD9-dependent response to PSG17. Additionally, we examined whether the tetraspanin CD81, which is closely related to CD9, can compensate for the absence of CD9 in macrophages, and whether CD9 is the receptor for more than one member of the murine PSG family. Our results are consistent with a role for PSGs as immune regulatory molecules that may influence maternal innate immune responses.

Methods

Animals and Cell Culture

RAW 264.7 cells were obtained from the American Type Culture Collection (Manassas, VA) and were cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose, 5 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B (PSA) and 10% fetal bovine serum (FBS). Chinese hamster ovary cells stably expressing PSG17N were maintained in Iscove's (Cellgro, Mediatech, Inc., Herndon, VA), 2% dialyzed FBS, 0.5 X PSA and 1.28 μM methotrexate (Calbiochem, La Jolla, CA). The cells were grown in a cellulose 4 kDa MW cutoff cartridge and the medium was harvested according to its lactate level (Spectrum Laboratories, CA).

Five- to six-week-old BALB/c mice were purchased from the NCI laboratories (Frederick, MD). C57BL/6 mice deficient in CD9 were bred from the CD9 +/- breeding pair received from Dr. Boucheix [24] and CD81-deficient mice were obtained after breeding CD81 +/- received from Dr. Levy [25]. All animals were placed in cages with filter tops and fed standard chow and water ad libitum according to NIH guidelines. Peritoneal macrophages were harvested and cultured as previously described [14]. Bone marrow-derived macrophages (BMDM) were isolated from the femur and tibia of wild type and CD9-deficient C57BL/6 mice. The cells were disaggregated by passage through an 18g needle, pelleted at 1000 rpm for 5 minutes, and red blood cells were lysed using NH_4Cl lysis solution (Sigma). After 5 minutes, 45 ml of PBS containing 2% FBS was added and the cells were pelleted. To generate an immortalized macrophage cell line, 2 x 10^7 bone marrow cells were resuspended in 5ml ψCREJ2 cell supernatant (a source of the

J2 transforming retrovirus) [26] with 500 μl of 500 μg/ml polybrene (Sigma) and 5 μl of 10^6 U/ml GM-CSF (Peprotech). After 24 hours at 37^{o}C, the supernatant was removed and the cells were grown in DMEM/ 10% FBS containing GM-CSF for 7 days. GM-CSF was then withdrawn and the cells were cultured further in DMEM/ 10% FBS to establish growth factor-independent cell lines. These cells were maintained in DMEM supplemented with 5% heat-inactivated FBS, 200 mM L-glutamine and PSA. For some experiments, when lower levels of FBS were desired, the cells were maintained in Advanced D-MEM (Invitrogen, Carlsbad, CA) with 2 % heat inactivated FBS.

FACS analysis

BMDM were subjected to analysis with an EPICS XL-MCL flow cytometer (Beckman Coulter) using the KMC8.8 PE-labeled anti-CD9 mAb (BD Pharmingen, San Diego, CA) and FITC-conjugated anti-F4/80 mAb (Serotec, Raleigh, NC). The data were collected for 1 x 10^4 cells. Prior to incubation with the specific Abs, the cells were incubated with 1 μg of Fc block (BD Pharmingen) per 1 x 10^6 cells.

Generation of recombinant proteins

To generate the recombinant PSG17N-Myc-His protein, the N-terminal domain of PSG17 was amplified by PCR from full length PSG17 [8] in pBluescript II KS+ (Stratagene, La Jolla, CA) with primers 5' GAAGATCTAGAG ATATGGAG(T/G)TGTC3' (underlined *Bgl*II site) and 5' TTGGTACCCTCATTT (A/G)TCACAG(C/T) CAGG 3' (underlined *Kpn* I site). The PCR product was inserted into the pCRII-TOPO vector (Invitrogen). PSG17N was removed from pCRII-TOPO by

digestion with *Bgl*II and *Kpn*I and ligated into the pcDNA3.1 Myc-His vector (Invitrogen). For the detection and purification of the protein, the myc-epitope and 6x histidines were added to the C- terminus. PSG17N-Myc-His was then inserted into the *Not*I- and *Pst*I-digested pFastBac (Invitrogen). Recombinant baculovirus was obtained following manufacturer's instructions (Invitrogen) and purified from insect cell supernatant as previously described for PSG18N-Myc-His [14]. Recombinant PSG19N-Myc-His was obtained by cloning PSG19N into pcDNA3.1 Myc-His prior to subcloning into pFastBac as described above. The GST-His-XylE control protein was purified as previously described [14] using glutathione-sepharose beads (Amersham).

The initial characterization of the activity of PSG17N in RAW cells and BALB/c peritoneal macrophages was performed using the recombinant protein produced in insect cells. Subsequent studies were performed using PSG17N-Myc-His secreted from CHO cells. To generate PSG17N-Myc-His in CHO cells, PSG17N-Myc-His was excised from pcDNA3.1 Myc-His with PmeI and subcloned into the EcoRV site of the pEAK10 vector (Edge Biosystems, Gaithersburg, MD). $DHFR^-$ CHO cells were co-transfected using the Fugene 6 reagent (Roche, Indianapolis, IN) with PSG17N-Myc-His in pEAK10 and pDHIP at a 10:1 molar ratio as previously described [27]. The pDHIP plasmid, which confers methotrexate resistance, was provided by Dr. G. Kaplan (FDA). A single cell clone expressing the highest level of PSG17N-Myc-His was selected for amplification and inoculation in a Cell Max cartridge (Spectrum Laboratories, Rancho Dominguez, CA).

PSG17N-Myc-His and PSG19N-Myc-His harvested from the supernatant of Sf9 insect cells grown in SF900 II media (Invitrogen) with 2% FBS or PSG17N-Myc-His harvested from the CHO cell medium were purified using identical conditions. The supernatant was first dialyzed in 160 x volumes of 300 mM NaCl and 50 mM NaHPO4 pH 7.4 overnight at 4^{o} prior to the incubation with Ni-NTA agarose beads (Qiagen, Valencia, CA) followed by washes and a final elution with 250mM imidazole pH 7.3 as per manufacturer's recommendations. The eluted proteins were then loaded onto a 10-11% SDS-PAGE in a prep cell 491 apparatus (Bio-rad, Hercules, CA) and fractions were collected as they migrated out of the gel, and were analyzed by immunoblot. To remove the SDS, fractions were electroeluted using a 2kDa MW cutoff cellulose acetate membrane in 0.01M Tris.HCl pH 8 (Harvard Apparatus, Holliston, MA). Prep cell fractions that reacted with both R90 pAb (see antibodies and reagents section) and anti-myc mAb (Invitrogen), were pooled together, concentrated using a Centriprep 10 (Millipore, Bedford, MA), dialyzed against PBS, and quantitated against BSA standards (Pierce, Rockford, IL) after Coommassie blue staining of the SDS-PAGE separated proteins. Fractions that contained proteins that did not react with R90 and anti-myc, were pooled together as well, their concentration adjusted to match that of the R90 and anti-myc-reactive fractions, and used as a control for the CHO cell produced PSG17N-Myc-His. All recombinant proteins were tested for endotoxin contamination using the *Limulus* amebocyte lysate assay (Biowhittaker,Woburn, MA). When endotoxin was detected, the proteins were further incubated with the ActiClean Etox resin (Sterogene Bioseparations, Carlsbad, CA), for its removal. Only recombinant proteins with no detectable LPS were used for all experiments.

Antibodies and reagents

We generated R90 by immunizing a rabbit with PSG17N-Myc-His produced in CHO cells using standard methodology (Southern Biotec, Birmingham, AL). For immunoblot analysis, the following Abs were employed: anti-myc (Invitrogen)**,** anti-Cox 2 polyclonal Ab (Upstate Biotechnology, Lake Placid, NY)**,** and anti-GST mAb (Santa Cruz Biotech, CA). Expression of CD81 in CD9 wild type and CD9-deficient BMDM was confirmed with a PE-labeled mAb TAPA-1 (eat2) (BD Pharmingen). The COX-2 specific inhibitor NS-398 (Cayman Chemicals, Ann Arbor, MI) was added to the cells at a concentration of 1 μM, 30 min prior to PSG17N or control protein addition. The protein kinase A inhibitor KT5720 (Calbiochem, La Jolla, CA**)** was added to the cells at a concentration of 50 nM for 6 hours before the addition of PSG17N or control protein. The neutralizing anti-TGF-β1/1.2 polyclonal chicken IgY Ab (R &D Systems, Minneapolis, MN) and the control Ab of the same isotype were added at 50 μg/ml for 30 min prior to PSG17N treatment.

Cytokine and PGE_2 ELISA

RAW 264.7 cells and peritoneal macrophages were seeded in 24-well tissue culture plates at 1 X 10^6 or 1.5 X 10^6 cells per well, respectively. BMDM were seeded at a density of 1.2 x 10^6 cell per well. After 24 h, the cells were treated in triplicate with PSG17N-Myc-His or the control protein for 4 h at 37^{o}C in 300 μl of media. When the treatments were for less than 2 hours, the volume was kept to 300 μl; after four hours, the volume was increased to 1 ml in each well by addition of cell culture media. Supernatants were harvested at different times post treatment depending on the cytokine under study.

ELISA was used to measure secreted IL-10, IL12p40, IL-6 (Pierce-Endogen, Woburn, MA), and TGF-β_1 (R & D Systems) in cell supernatants. The limit of detection was 31 pg/ml for IL-10 and 15 pg/ml for IL-6, IL-12p40, and TGFβ_1. PGE_2 secretion was determined using the high sensitivity (8.26 pg/ml) ELISA kit (Assay Designs, Ann Arbor, MI). Prior to measuring IL-10 production by BALB/c thioglycollate-elicited peritoneal macrophages, the supernatant was concentrated 2.5 fold with a microcon YM3 concentrator (Millipore). For measuring IL-10 production from macrophages obtained from mice of the C57BL/6 background, the supernatants were concentrated 8-10 fold prior to the ELISA or the cells were treated with suboptimal concentrations of LPS (1ng/ml) at the time of treatment with PSG17N or the control protein. Treatment with 0.1-1 μg/ml of LPS was used as a positive control.

Semi-quantitative RT-PCR

RAW 264.7 cells grown in a 24-well plate were treated with 15 μg/ml recombinant PSG protein or the control protein GST-His-XylE. Cycloheximide (Sigma, St. Louis, MO) was added at a concentration of 5 μg/ml where indicated. Total RNA was harvested at 2 or 4 h post treatment using TRIzol (Invitrogen) according to manufacturer's instructions. RNA was reverse transcribed using random hexamers and Ready-to-go you-prime-first strand beads (Amersham). One tenth of the reverse transcriptase reaction was used for PCR amplifications of IL-10, IL-6, or GAPDH. Various PCR cycles were analyzed to optimize the linear correlation between RNA and PCR product. Amplified products were electrophoresed on a 1.5% TBE agarose gel and blotted onto Nytran membranes. Following UV cross-linking, the membranes were

incubated with specific internal ^{32}P- labeled oligonucleotide probes. After hybridization, the band intensity was quantified using the Storm Phosphorimager and Image QuaNT program (Molecular Dynamics, Sunnyvale, CA). IL-10 and IL-6 cDNA intensity measurements were normalized to the GAPDH PCR product value [28].

Immunoblot analysis

BMDM lysates were obtained by adding 120 μl of phosphosafe buffer (Novagen, Madison, WI) with protease inhibitors to cells seeded at a density of 5 x 10^6 cells/well of a 6-well plate the previous day. The protein concentration in each well was determined using the Coomassie plus reagent (Pierce) and equal amounts (100 μg) were loaded per lane of a 4-20% NuPage gel (Invitrogen). After transferring to a PVDF membrane and blocking, the membranes were incubated overnight at 4^{o}C with the specific Ab at the concentration recommended by the manufacturer. For analysis of COX-2 induction, an anti-GAPDH mAb (Research Diagnostics, Flanders, NJ) was used for loading normalization.

Protein-Protein interaction assay

The GST-mouse CD9 extracellular loop 2 fusion protein (GST-CD9EC2), wild type and triple mutant, were generated as previously described [29]. Purified recombinant PSG19N-Myc-His was tested for its ability to bind to GST-CD9EC2 with the pull-down poly His protein:protein interaction kit (Pierce) following manufacturer's recommendations. Briefly, 100 μg of PSG19N-Myc-His was allowed to bind to the cobalt chelate gel and after 5 washes 100 μg of GST-CD9EC2 or GST-CD9TM or GST-

CD63, a tetraspanin family member used as control, was added to the gel and incubated overnight with gentle rocking at 4^{o}C. After 7 washes, the proteins were eluted with the imidazole elution buffer and 25 μl of the eluted material was loaded on a 4-20% NuPage gel. Proteins were detected by immunoblotting with the use of an anti-GST mAb followed by horseradish peroxidase-conjugated goat anti-mouse antibody and the Super Signal chemiluminescent detection system (Pierce).

Data analysis

Data were obtained from at least three independent experiments. Results were evaluated for statistical significance using the unpaired Student's *t* test. Data were expressed as mean ± standard error (S.E.) and significance was defined at $p < 0.05$.

Results

PSG17 induces secretion of IL-6, IL-10, and TGFβ1 in murine macrophages in a dose dependent manner

We previously reported that recombinant PSG1, 6, and 11 induced IL-6, IL10, and TGFβ1 in human monocytes [12]. In addition, we reported that PSG18N induced IL-10 in RAW 264.7 cells [14]. To determine whether murine and human PSGs have similar biological effects, RAW 264.7 cells and BALB/c thioglycollate peritoneal macrophages were treated with increasing concentrations of recombinant PSG17N-Myc-His or a control protein, GST-His-XylE, and cytokines in the supernatant were measured at 24 h post-treatment for IL-10 and IL-6 and 2 h post-treatment for TGFβ_1. Treatment of the cells with 20-25 μg/ml of PSG17N resulted in significant secretion of IL-10 and IL-6 when compared to the levels of these cytokines obtained following treatment with the control protein [30] (Waterhouse R., Dissertation, 2001). Up-regulation of TGFβ_1 was observed with concentrations of PSG17N as low as 2.5 (for RAW 264.7 cells) or 5 μg/ml (for primary macrophages) and was observed at 2 h post-PSG17 treatment. The observed increase in secretion of these cytokines was not a result of earlier macrophage production of pro-inflammatory cytokines, as supernatants from PSG17N-treated macrophages did not show an increase in TNF-α or IL-12 measured up to 48 h post treatment (Waterhouse R., Dissertation, 2001).

Induction of IL-10, IL-6, and TGFβ_1 by PSG17 is CD9 mediated.

We identified the receptor for PSG17 in macrophages as the tetraspanin CD9 by

screening an expression library [15]. Our next objective was to determine whether the observed biological responses to PSG17 treatment were CD9-mediated. In addition, we examined whether the absence of CD81, a tetraspanin known to complex with CD9, could interfere with the ability of PSG17 to stimulate the production of immuno-regulatory molecules in murine macrophages.

Because CD9-deficient mice are of the C57BL/6 background, we repeated the studies performed in the RAW cells and BALB/c peritoneal macrophages in macrophages isolated from C57BL/6 wild type and CD9-deficient mice. Figure 1A-B-C shows that PSG17N induced secretion of IL-10, IL-6, and TGFβ_1 in thioglycollate-elicited macrophages isolated from wild type mice. However, in macrophages isolated from CD9-deficient mice the level of all three cytokines was the same in the control and PSG17N-treated cells. Thus, induction of anti-inflammatory cytokines by PSG17N is blocked in the absence of CD9. Wild type and CD9-deficient macrophages, while responding equally to LPS treatment (Figure 1E), did not up-regulate IL-12p40 in response to PSG17N treatment (Figure 1D).

CD9-deficient females are infertile [24] and the litter size of CD9+/- mice is small, therefore, we used the J2 retrovirus (myc/raf) to generate immortalized bone marrow-derived macrophages (BMDM) from both wild type and CD9-deficient mice for further characterization of the PSG17-CD9 mediated responses. FACS analysis was employed to verify the CD9 phenotype of the immortalized cells and to confirm their macrophage lineage using the anti-CD9 KMC8.8 and the anti-F4/80 mAbs, respectively (Dveksler, private communication). The derivation of these cells allowed us to study their responses to PSG17N over a range of concentrations and time points. PSG17N did not

induce the expression of IL-6, IL-10, and TGFβ_1 in CD9-deficient BMDM at any concentration examined. As expected, PSG17N did not induce IL-12p40 in wild type or CD9-deficient BMDM (Figure 2). Importantly, as primary macrophages, wild type and CD9-deficient BMDM secreted an equal amount of IL-10, IL-6, and IL-12p40 in response to LPS treatment (Dveksler, private communication).

These results suggest that other tetraspanin family members, which are present in complex with CD9, do not compensate for the lack of CD9 in macrophages since high concentrations of PSG17N were unable to initiate cytokine signaling in these cells. To expand on this observation, we investigated whether the absence of CD81 expression had an effect on the PSG17-mediated expression of anti-inflammatory cytokines. Peritoneal macrophages isolated from BALB/c wild type and CD81-deficient mice responded equally to the treatment with PSG17 and LPS as well (Figure 3). In light of this observation, it is important to note that BMDM expressed CD81 even in the absence of CD9 (Dveksler, private communication).

CD9 is the receptor for PSG19

To further characterize the involvement of CD9 in the response to murine PSGs, we set out to clone the receptor for PSG19, a member of the murine PSG family. Our initial binding experiments performed using ELISA and FACS analysis did not clearly demonstrate whether PSG19 bound to CD9. For that reason, we decided to clone the receptor for PSG19 by screening an expression library as previously reported for the identification of the PSG17 receptor [15]. Briefly, following four rounds of panning of transfected HEK 293T cells onto PSG19N-coated Petri dishes, we recovered the cells that bound to PSG19N. The plasmids isolated from these cells were amplified and sequenced.

The cDNA sequence of these plasmids was identical with that of CD9. Binding of PSG19N to CD9-EC2 wild type but not CD9-EC2 triple mutant [SFQ(173-175)AAA] was confirmed by protein-protein interaction assay as illustrated in Figure 4.

PSG17 induces Prostaglandin E_2 and up-regulates COX-2 expression

Upregulation of IL-6 and IL-10 mRNA in RAW cells after treatment with PSG17 requires *de novo* protein synthesis, as demonstrated by the significant inhibition in the expression of these cytokines upon treatment of the cells with 5 μg/ml of cycloheximide [30]. Prostaglandin (PGE_2) has been shown to play a role in regulating the Th1/Th2 balance [31] [32]. [32]. This prompted us to investigate whether PSG17N can induce PGE_2 secretion and, as a result, has the ability to potentially regulate both the innate and acquired immune responses. RAW cells were treated with 10 μg/ml PSG17N. In response to PSG17N significant levels of PGE_2 were produced (Figure 5A). Supernatants analyzed at 2, 4, and 6 h post treatment showed that PGE_2 secretion peaked at 2 h post treatment, and at later time points was no longer significant compared to treatment with the control protein [30]. When PGE_2 levels were measured in BMDM supernatants, they were up-regulated in response to PSG17N and reached a maximal concentration at 6 h post treatment. PGE_2 secretion in response to PSG17N treatment was observed only in wild type mice, which correlated with the induction of COX-2 expression, the rate limiting catalyzing enzyme involved in PGE_2 synthesis (Figure 5B and C).

Induction of IL-10 and IL-6 requires induction of PGE_2 secretion.

The finding that PGE_2 enhances the production of IL-10 and IL-6 protein levels by activated murine macrophages suggested that PGE_2 elevation following COX-2 expression could be required for the observed induction of IL-10 and IL-6. BMDM were pre-incubated with the COX-2 specific inhibitor NS-398 prior to treatment with PSG17N and the control protein. As shown in Figure 5B, the drug inhibited the production of PGE_2 in response to PSG17N and it reduced the induction of IL-10 and IL-6 in a significant manner (Figure 6A). The completely inhibitory production of LPS-induced IL-10 and IL-6 by NS-398 was demonstrated in Figure 6C.

Binding of PGE_2 to its specific G-protein coupled receptors, i.e., EP2 and EP4, generates intracellular cAMP, a molecule absolutely required for the activation of protein kinase A [33] [34]. Phosphorylation of PKA on its downstream transcriptional factors, such as CREB, induces the expression of target genes [35, 36, 37]. To study the involvement of the cAMP-dependent PKA pathway in PSG17N-mediated cytokine production, the effect of PSG17N on BMDM was evaluated in the presence or absence of KT5720, a cAMP-dependent PKA inhibitor. As shown in Figure 6B, the introduction of this inhibitor significantly reduced the induction of IL-10 and IL-6. On the other hand, treatment of cells with NS-398 or KT5720 had no effect in the induction of $TGF\beta_1$ (Dveksler, private communication). The PKA inhibitor partly reduced IL-6 and IL-10 induced by LPS (Figure 6C).

$TGF\beta_1$ has been reported to induce IL-6 production in peripheral blood mononuclear cells, and up-regulate IL-10 production in murine macrophages, which may explain the significance of early production of this cytokine by activated macrophages

[38] [39]. We, therefore, investigated whether neutralization of TGFβ_1 had an effect on IL-6 and IL-10 induction. No difference in the levels of PSG17N-mediated IL-6 and IL-10 was observed in BMDM co-treated with anti-TGFβ Ab or with an isotype match Ab control, or in the cells treated with PSG17N only (data not shown).

Discussion

Experimental evidence indicates that various mechanisms, not mutually exclusive, are potentially responsible for fetal tolerance [40]. The mechanism most relevant to the data we present here is the development, by the maternal immune response during pregnancy, of a bias toward a Th2 or anti-inflammatory environment [41]. A Th2 immune environment is characterized by the presence of specific cytokines including IL-10, TGF-β_1 and IL-4. These cytokines reduce the expression of pro-inflammatory cytokines, which are known to be detrimental to pregnancy [42].

Macrophages respond to various non-self stimuli by secreting cytokines and, as such, are an essential component of the innate immune system. These cells are affected by various circulating factors produced during pregnancy [43]. In this study, murine macrophages isolated from wild type and CD9- or CD81-deficient mice were treated with highly purified recombinant PSG17N. Our results show that PSG17N induced the secretion of IL-6, IL-10, TGFβ_1, and the eicosanoid PGE_2, and that the induction of these cytokines by PSG17 required CD9 expression. We have recently found that besides binding to macrophages, PSG17 binds to bone marrow-derived dendritic cells, which express CD9. Other immune cells that express CD9 include T cells, marginal zone B cells, B1 cells, and plasma cells [44] [45]. Expression of CD9 in human decidual NK cells has been recently described [46]. Interestingly, Motran and colleagues have reported that human PSG1a induces a state of alternative activation of macrophages that results in the inhibition of mitogen-induced proliferation of naïve T cells [47]. We are currently

investigating whether PGS17 can, upon binding to CD9, influence cytokine secretion and proliferation of T cells in response to specific antigens.

Tetraspanins are expressed together as part of a multimolecular complex on the cell surface [48]. In some instances one tetraspanin could compensate for the lack of expression of the other. This is the case for CD81, which has been shown to compensate for the deficiency of CD9 in egg-sperm fusion [49], whereas, the unique interaction between CD81 and the envelope protein of hepatitis C virus reveals a specific function for CD81 [50] [48]. In this investigation, we demonstrated that this seems to be the case for CD9 being the unique receptor for PSG17. In previous studies, we were unable to detect binding of PSG17 to any of the other tetraspanins tested [15] and here we confirmed that the lack of the CD9 partner CD81 does not alter the response of macrophages to PSG17N. Recently we reported that the amino acids SFQ (173-175) in the extracellular loop 2 of CD9 are important for the binding of PSG17 to CD9 [16]. This sequence is not present in the extracellular loop 2 of CD81, which suggests that CD81 not involved in the cytokine induction that is mediated by PSG17N. This is in agreement with our findings.

Although fold induction of cytokines IL-10, IL-6, PGE_2, and $TGF\beta_1$ in response to PSG17N treatment was different in RAW, thioglycollate-induced BALB/c and C57BL/6 peritoneal macrophages, and BMDM, it is clear that these cytokines were induced by PSG17N. Through induction of IL-10, PSGs may contribute to the down-regulation of inflammatory Th1 cytokines locally at the fetal maternal junction and/or systemically. Increased concentrations of IL-10 have been reported at the fetal-maternal interface in normal pregnancies in mice and humans [51] while decreased concentrations

of IL-10 in serum have been associated with pre-eclampsia and spontaneous abortion [13] [52] [53]. IL-6 is a multi-functional cytokine with the ability to stimulate a variety of different cells and has been demonstrated to function as an important anti-inflammatory cytokine both locally and systemically [54]. Uterine decidual cells and macrophages have been reported to express IL-6 during implantation and throughout pregnancy in mice [55,56, 57]. Production of this cytokine inhibits IL-1 and TNFα production by macrophages [58]. These reports suggest that the induction of IL-6 by PSGs in macrophages may also play important roles in placental regulation of maternal immune responses and in placental development. In addition to its role during the immune response, IL-6 induces production of human chorionic gonadotropin from first-trimester human trophoblasts in culture [59].

$TGF\beta_1$ secretion was upregulated within 2 h of PSG17 treatment in thioglycollate-induced and J2 transformed BM-derived macrophages, while significant induction of this cytokine was not detected at 24 h post treatment as it was for PSG17-treated RAW264.7 cells. Although PSG17 induced both PGE_2 and $TGF\beta_1$ secretion in all the macrophage populations studied, the kinetics of induction in RAW cells differed from that observed in BMDM and peritoneal macrophages. It is possible that the observed kinetics reflects differences in the activation state of the cells.

The lack of induction of IL-12 and TNF-α by PSG17 concurs with our previous data on macrophage treatment with human PSG1, 6, and 11 and murine PSG18, none of which increased secretion of inflammatory cytokines at the RNA or protein level. The absence of Th1 type cytokines in response to PSGs suggests that these proteins are just non-specifically activating macrophages, but are inducing a specific response.

PGE_2 has been reported to regulate IL-6 and IL-10 production by activated macrophages, and to regulate T cell differentiation, favoring Th2 T cell development, through induction of IL-10 [32] [60]. In BMDM, COX-2 induction was observed starting at 2 h post 17N treatment followed by an increase of PGE_2 secretion detected at 6 h post treatment. Inhibition of COX-2 resulted in a significant decrease in the PSG17N-mediated up-regulation of IL-10 and IL-6 but it did not affect the secretion of $TGF\beta_1$.

We next studied the involvement of the cAMP-dependent PKA pathway in the PSG17N-mediated induction of IL-10 and IL-6. We found that the introduction of the cAMP/PKA selective inhibitor KT5720 significantly reduced the secretion of IL-10 and IL-6. Most of the genes regulated by cAMP/PKA contain a cis-acting DNA sequence, named CRE (cAMP-responsive element), which is the binding site for the transcriptional factor, CREB (CRE-binding protein). cAMP-dependent PKA phosphorylation of ser133 on CREB is essential for its binding to CBP/p300, which in turn connects the activator complex to a basal transcriptional machinery [61]. Interestingly, the COX-2, IL-10, and IL-6 genes have CRE sites in their 5' flanking promoter region [62] [37] [63] [64]. Preliminary experiments to examine the effect of PSG17N on CREB phosphorylation showed that phosphorylated CREB could be detected 15 min post PSG17N treatment only in wild type but not in CD9-deficient BMDM. Future studies are needed to determine the duration and magnitude of the phosphorylation of CREB and the regulation of other transcription factors in response to PSG17N.

It has been reported that TGFβ up-regulates IL-10 and IL-6 in various cell lines [38] [65] [39]. Recently, TGFβ has been shown to activate cAMP-independent PKA via the interaction of Smad3/Smad4 with the PKA regulatory subunits and to mediate the

subsequent induction of CREB phosphorylation [66]. Hence, we decided to investigate the effect of PSG17N-mediated secretion of TGFβ_1 on IL-10 and IL-6 production. However, we were unable, via neutralization of TGFβ_1 during PSG17N treatment, to demonstrate a relationship between TGFβ1 secretion and the up-regulation of IL-10 and IL-6.

Published studies suggest that in macrophages elevated intracellular cAMP results in extracellular signal-regulated kinase (ERK) activation [67]. In addition, Williams and colleagues reported that the synthesis of IL-10 in response to PGE_2, is regulated by p38 mitogen-activated protein kinase (MAPK) in inflammatory macrophages [60]. There is accumulative evidence that MAPK family activation correlates with COX-2/PGE2 up-regulation and, as a result, increases the production of IL-6 [68] [69]. Furthermore, Giroux reported that PKC-α modulates COX-2 expression in macrophages exposed to both LPS and IFN-γ although the mechanism by which this occurs was not identified [70]. Interestingly, CD9 has been shown to associate with activated PKC in Jurkat and K562 cells [71]. In light of these findings, the relationship between the MAPK family and PKC, on the one hand, and the activation of PSG17N-mediated COX-2/PGE_2 pathway, which has been shown to be a major regulator of the synthesis of IL-10 and IL-6, on the other, merits further investigation.

In conclusion, our results indicate that the biological effect of murine PSG17 is similar to that of three human PSG family members in macrophages isolated from Th1-dominant (C57BL/6) and Th2-dominant (BALB/c) mice. Importantly, the tetraspanin CD9 is required for the PSG17-mediated cytokine induction and is the receptor for another member of this family, PSG19, which raises the possibility that all murine PSGs

share the same receptor. Preliminary data from our laboratory reveal that PSG19, like PSG17, induces the secretion of the same cytokines in macrophages. In contrast, they also show that human PSG1d and 11 do not use CD9 as their receptor. In this respect, it is important to note that members of the human and murine PSG family have only 60% homology in the N-terminal domain, which we have shown is sufficient for the ability of these proteins to induce cytokines. On the other hand, the homology between human PSGs is greater than 85%, which suggests that they might use the same receptor. Through binding to different receptors, human and murine PSGs may utilize identical signaling mechanisms that result in the secretion of the same cytokines. The identity of the receptor for human PSGs as well as the signaling molecules involved remains to be elucidated.

High levels of PSG expression in human pregnancy and decreased production in fetal pathologies imply a critical role for these proteins in pregnancy. We suggest that the induction of anti-inflammatory mediators in macrophages by placentally produced PSGs plays a role in the generation of an immune environment compatible with successful pregnancy.

Acknowledgements

We are grateful to Kimberly White, Carolyn Zalepa, Julie Wu, and Karen Wolcott for technical assistance and to Dr. G. Kaplan (CBER, FDA) for providing us with the pDHIP vector. In addition, we are most grateful to Dr. Boucheix and Dr. Rubinstein (Hopital Paul Brousse, Villejuif, France) for providing us with the CD9 +/- mice and for sharing their expertise in tetraspanins. We thank Dr. Shoshana Levy (Division of Oncology, Stanford University School of Medicine, Stanford, CA) for providing us with CD81 +/- mice and critical comments. We would like to thank Dr. Wolfgang Zimmerman (Tumor Immunology Laboratory, University Clinic Grosshadern, Ludwig Maximilians University Munich, Germany) for providing us with the PSG17 cDNA.

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Figure Legends

Figure 1. PSG17N induces IL-10, IL-6, and TGFβ1 secretion in C57BL/6 thioglycollate-elicited peritoneal macrophages expressing CD9. Peritoneal macrophages isolated from wild type and CD9-deficient (CD9KO) mice were treated in triplicate with PSG17N-Myc-His, the control protein GST-His-XylE or LPS (100 ng/ml). Supernatants were harvested at 24 h post treatment (A, B, D, and E) or at 2 h post treatment (C). PSG17N and the control protein were added at a concentration of 25 μg/ml for the analysis of IL-10, IL-12, and TGFβ1, and 15 μg/ml for the analysis of IL-6. For IL-10 quantitation, the cells were co-treated with PSG17N and a sub-optimal concentration of LPS (1ng/ml) and therefore measured in different supernatants than IL-6. The results are expressed as the means $\pm$ SEM and are representative of three experiments. *$p<0.05$ compared with control treated cells.

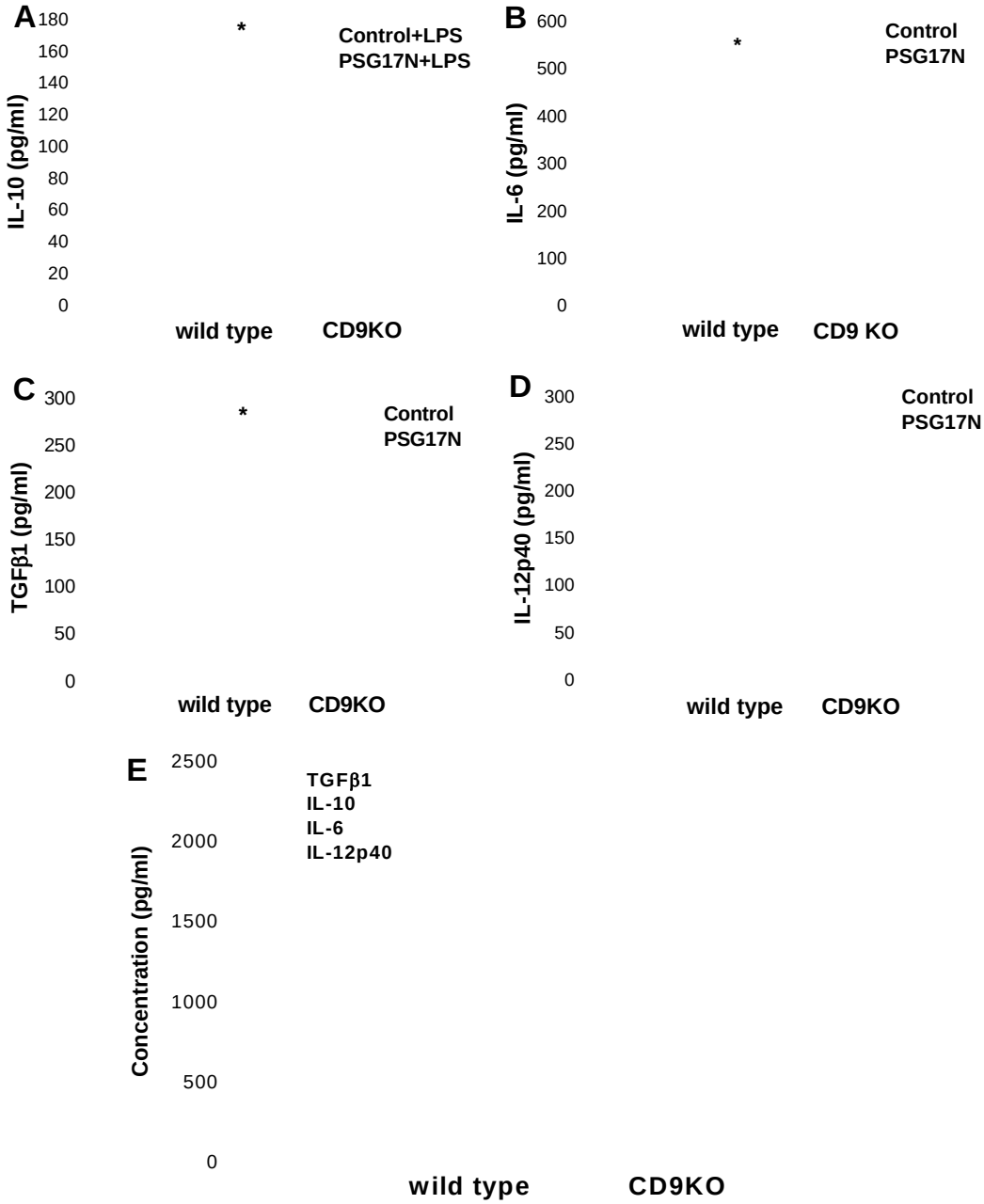
Figure 2. CD9 is required for the dose-dependent induction of IL-10, IL-6, and TGFβ$_1$ by PSG17 in BMDM. J2 transformed bone marrow derived macrophages (BMDM) from C57BL/6 wild type and CD9-deficient (CD9KO) mice were treated in triplicate with PSG17-Myc-His or the control protein, and the cytokines in the supernatants were measured by ELISA. As described in Figure 1, IL-10 production was measured in response to PSG17N and 1ng/ml of LPS treatment. IL-6 and IL-12 were analyzed in the supernatants at 24 h post treatment while TGFβ1 was measured at 2 h post treatment. All data are representative of three experiments. *$p<0.05$ compared with control treated cells.

Figure 3. CD81 is not required for PSG17-mediated cytokine secretion. Thioglycollate-elicited peritoneal macrophages isolated from BALB/c wild type (CD81WT) and CD81 deficient (CD81KO) mice were treated in triplicate wells with 25 μg/ml of PSG17N-Myc-His or the control protein. Supernatants were analyzed as described in the Figure 1 legend.

Figure 4. PSG19N binds to the extracellular loop 2 of CD9. Cobalt chelate beads were pre-incubated with PSG19N-Myc-His followed by GST-hCD63EC2 (lane 1) or GST-CD9EC2 (lane 2) or GST-CD9-TM (lane 3). After several washes, the proteins were eluted with a buffer containing 290 mM imidazole and separated on a 4-20% NuPage gel. Co-immunoprecipitation of PSG17N and CD9EC2/CD9-TM was detected by Western blot using an anti-GST mAb.

Figure 5. The PSG17-CD9 mediated induction of COX-2 results in the secretion of PGE_2. (A). RAW cells were treated with PSG17N-Myc-His or the control protein in triplicate and the supernatants were harvested at 2 h for the analysis of PGE_2 by ELISA. (B). C57BL/6 wild type and CD9-deficient BMDM were treated with control protein or PSG17N-Myc-His in the presence or absence of NS-398. At 6 h post treatment, PGE_2 was measured in the supernatants by ELISA. (C) BMDM from wild type and CD9-deficient mice were treated with 20 μg/ml of PSG17N-Myc-His, control protein or LPS (1 μg/ml) for 6 h. Cell lysates were analyzed for the expression of COX-2 and GAPDH by Western blot.

Figure 6. IL-10 and IL-6 secretion requires induction of COX-2 and activation of protein kinase A. C57BL/6 BMDM were treated in triplicate with 20 μg/ml of PSG17N-Myc-His or control protein in the presence or absence of the COX-2 inhibitor NS-398 (A) and the cAMP-dependent PKA inhibitor KT 5720 (B). LPS was used at 100 ng/ml as the control (C). The supernatants were harvested at 24 h post treatment and IL-6 and IL-10 were measured by ELISA. For IL-10, the supernatant was concentrated 8 fold prior to ELISA. All data are representative of three experiments. *$p<0.05$ compared with PSG17N and inhibitor co-treated cells.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Control+LPS
PSG17N+LPS
wild type
CD9KO
B
IL-6 (pg/ml)
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PSG17N
wild type
CD9 KO
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TGFβ1 (pg/ml)
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PSG17N
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CD9KO
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IL-12p40 (pg/ml)
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Control
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wild type
CD9KO
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Concentration (pg/ml)
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IL-6
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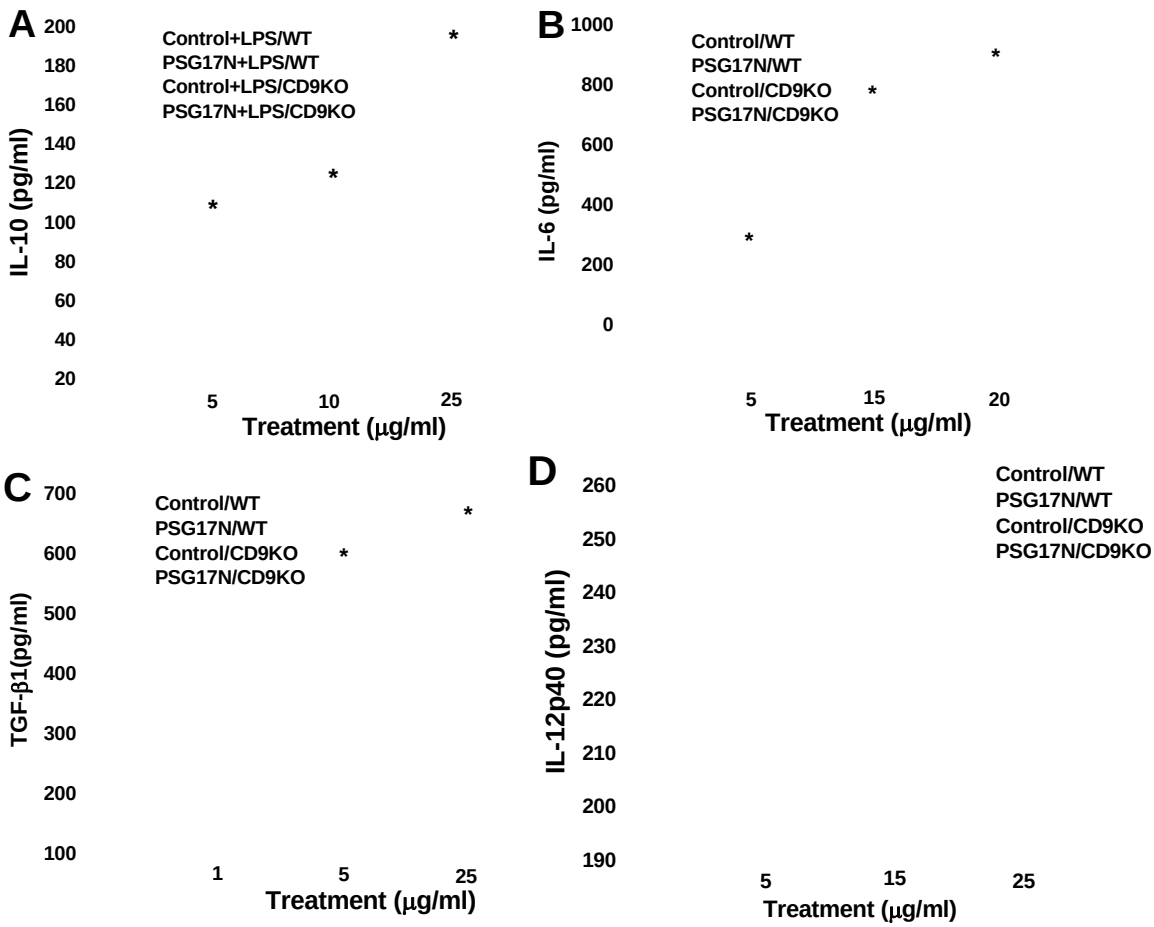
wild type
CD9KO

Figure 1.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PSG17N+LPS/WT
Control+LPS/CD9KO
PSG17N+LPS/CD9KO
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Treatment (μg/ml)
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800
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200
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Control/WT
PSG17N/WT
Control/CD9KO
PSG17N/CD9KO
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5
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Treatment (μg/ml)
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TGF-β1(pg/ml)
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500
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200
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Control/WT
PSG17N/WT
Control/CD9KO
PSG17N/CD9KO
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25
Treatment (μg/ml)
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IL-12p40 (pg/ml)
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250
240
230
220
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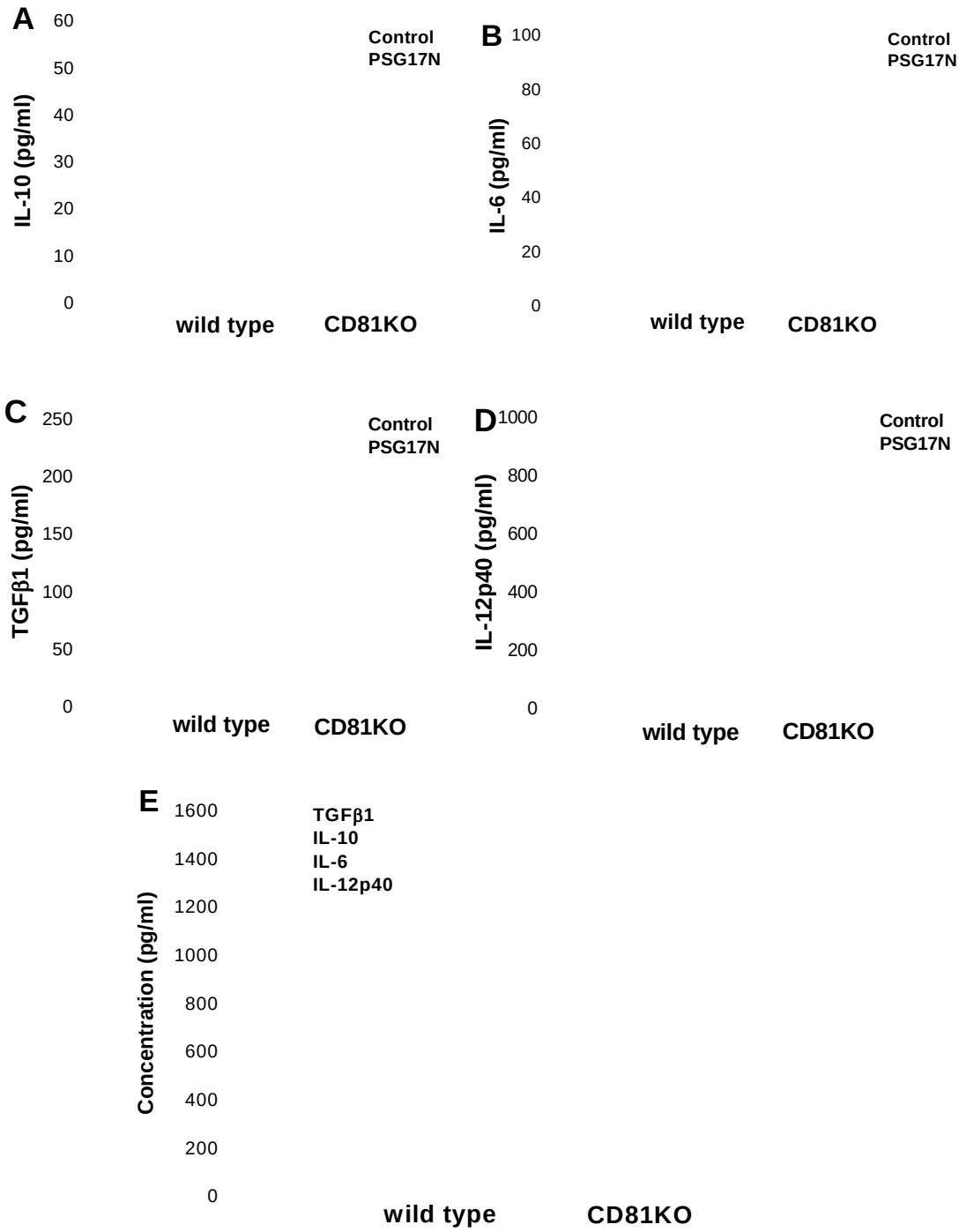
15
25
Treatment (μg/ml)

Figure 2.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Control
PSG17N
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CD81KO
C
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PSG17N
wild type
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IL-12p40 (pg/ml)
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Control
PSG17N
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Concentration (pg/ml)
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1400
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TGFβ1
IL-10
IL-6
IL-12p40
wild type
CD81KO

Figure 3.

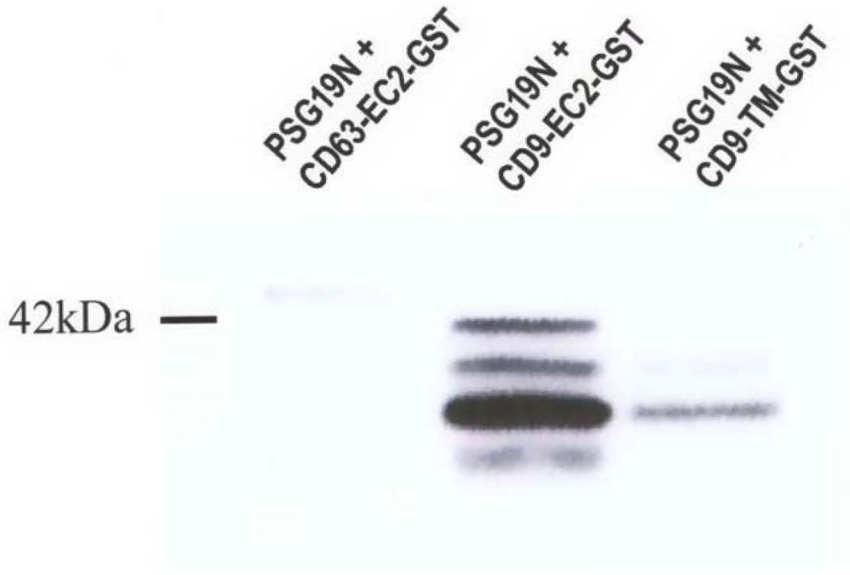
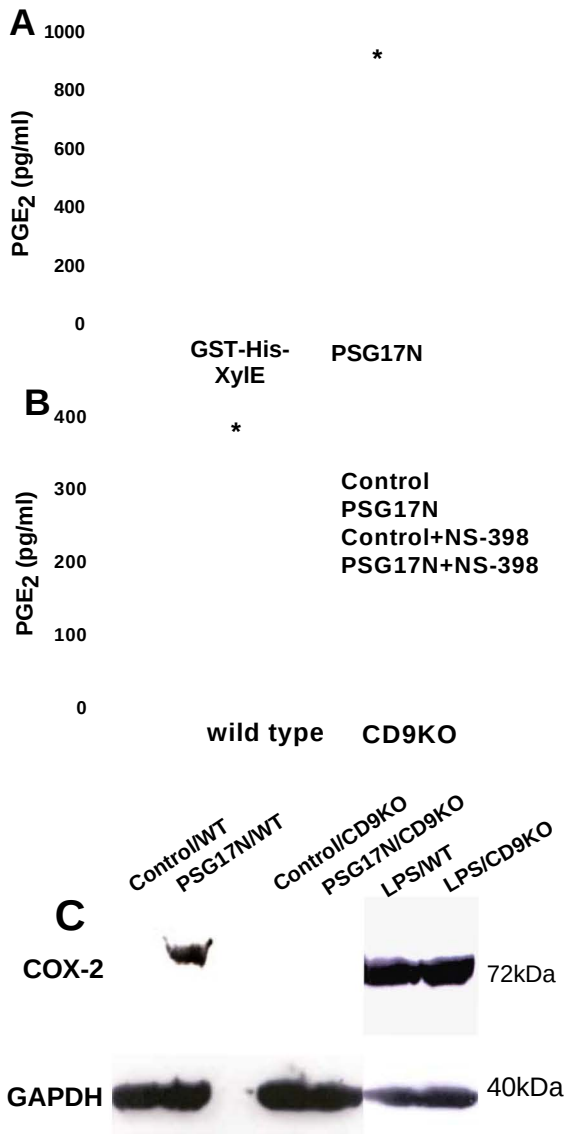

PSG19N +
CD63-EC2-GST
PSG19N +
CD9-EC2-GST
PSG19N +
CD9-TM-GST
42kDa

Figure 4.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800
600
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200
0
PGE2 (pg/ml)
*
GST-His-XylE
PSG17N
B
400
300
200
100
0
PGE2 (pg/ml)
*
Control
PSG17N
Control+NS-398
PSG17N+NS-398
wild type
CD9KO
Control/WT
PSG17N/WT
Control/CD9KO
PSG17N/CD9KO
LPS/WT
LPS/CD9KO
C
COX-2
72kDa
GAPDH
40kDa

Figure 5.

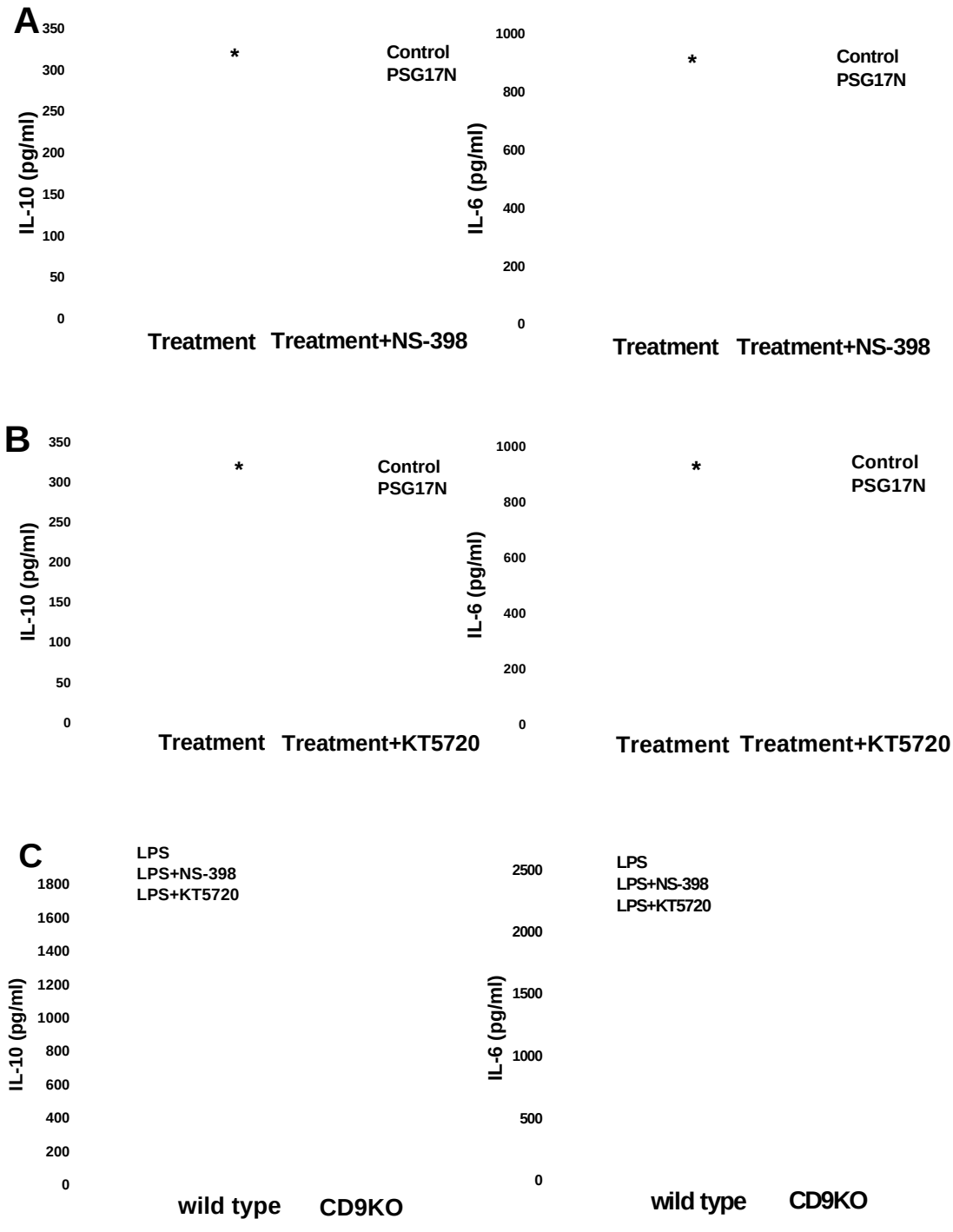
A
IL-10 (pg/ml)
350
300
250
200
150
100
50
0
*
Control
PSG17N
Treatment
Treatment+NS-398
IL-6 (pg/ml)
1000
800
600
400
200
0
*
Control
PSG17N
Treatment
Treatment+NS-398
B
IL-10 (pg/ml)
350
300
250
200
150
100
50
0
*
Control
PSG17N
Treatment
Treatment+KT5720
IL-6 (pg/ml)
1000
800
600
400
200
0
*
Control
PSG17N
Treatment
Treatment+KT5720
C
LPS
LPS+NS-398
LPS+KT5720
IL-10 (pg/ml)
1800
1600
1400
1200
1000
800
600
400
200
0
wild type
CD9KO
LPS
LPS+NS-398
LPS+KT5720
IL-6 (pg/ml)
2500
2000
1500
1000
500
0
wild type
CD9KO

Figure 6.

PART THREE

DISCUSSION

1 SUMMARY OF THE RESULTS

This investigation sought to add to our knowledge on the biological events induced by the ligand-receptor interaction of PSG17 and CD9. We also took some initial steps to examine the signaling pathways underlying the PSG17N-mediated cytokine induction in macrophages. The results demonstrate that CD9 expression is required for this pathway. More specifically, PSG17N binds directly to the large extracellular loop of CD9 and the residue phenylalanine 174 in this loop is essential for the interaction. Murine wild type but not CD9-deficient macrophages secrete IL-10, IL-6, PGE_2, and $TGF\beta_1$ in response to PSG17N treatment. We subsequently identified the signaling pathways that are involved in the PSG17N-mediated up-regulation of IL-10 and IL-6 in macrophages: COX-2-generated PGE_2 and cAMP-dependent PKA. And finally, CD9 was found to be also the receptor for murine PSG19.

These findings extend our knowledge of the mechanism that PSGs, an important contributor to the maintenance of maternal tolerance of the fetus, employ to induce macrophages to secrete a set of anti-inflammatory cytokines.

2 DISCUSSION

2.1 PSG17 binds to the large extracellular loop of CD9.

Tetraspanins are well known for their lateral association (in *cis*) with members of their own family or with various transmembrane-bound molecules including integrins, various immuno-receptors, the EWI family, and signaling molecules. While there are only a few soluble ligands, described as *trans* binding partners for tetraspanin members, there is evidence that the large extracellular loop (EC2) is the domain where the ligand-

receptor binding takes place [75] [76] (Figure 2B). In this present research, we demonstrated by pull-down assay a direct binding between the N1domain of PSG17 and the EC2 of CD9. The inability of CD9- EC2 to bind to the N terminal domain of CEACAM, a member of CEA subgroup, shows that the interaction of CD9-EC2 and PSG17N examined here is specific. This result is consistent with our previous observation showing that preincubation of CD9-expressing cells with the anti CD9 mAb, KMC8.8, inhibited the binding of PSG17N to these cells [21]. KMC8.8 has been shown to react with the EC2 [76] and results in macrophage activation upon co-cross linking of CD9 and the Fcγ receptors in macrophage cell lines [88]. However, treatment of macrophages with KMC8.8 did not induce cytokines in our study (data not shown).

The residues SFQ (173-175), which are located in the variable sub-domain of CD9-EC2, are required for egg-sperm fusion, as reported by Zhu et al. [76]. Injection of CD9 mRNA, but not CD9- SFQ (173-175) to AAA mRNA, into CD9-deficient oocytes restores the fusion ability [88]. While the binding partner for CD9 on the egg is still unknown, there is a corresponding region of CD9-SFQ in CD81-EC2 that mediates the binding of CD81 to the envelop protein of hepatitis C virus (in *trans* interaction) [75]. With pull-down assay, we determined that the SFQ residues are also required for the PSG17N and CD9 interaction. In addition, the phenylalanine 174 was found to be crucial for the binding as shown in FACS and ELISA. In data provided by Zhu, both mutated proteins, CD9-EC2- SFQ to AAA (triple mutant) and CD9-EC2-F174A (single mutant), generated by site-directed mutagenesis, have the correct folding. Additionally, when expressed in tissue culture cells, they are able to interact laterally with their major partner, CD9 P-1, also known as EWI-F, as does the CD9WT [76]. The fact that despite

exhibiting appropriate functionality and structure, the mutants are unable to bind to PSG17N, is further evidence for the specificity of the effect of the SFQ mutation on CD9-PSG17 interaction. These results prompted our collaborators to examine the effect of PSG17N on egg-sperm fusion, from which they found that pretreatment of eggs with PSG17N inhibited subsequent gamete fusion. In addition, injection of CD9-deficient eggs with the mRNA of wild type CD9 but not with the mRNA of mutated CD9 restored the egg-sperm fusion, indicating the SFQ (173-175) residues in the CD9-EC2 was also the active site for gamete fusion and PSG binding. On the basis of the findings, Ellerman proposed that the sperm might express a membrane-associated form of PSG17 or a related member of the CEA family that, upon binding to CD9 on eggs, initiates gamete fusion [Paper 1 of this dissertation]. A research project for the identification of that binding partner is currently underway.

2.2 CD9 is the only receptor required for cytokine induction mediated by PSG17 in macrophages.

Macrophages are an essential component of the innate immune system to defend against microbial infection and intracellular pathogen invasion. In response to various circulating factors, such as hormones, cytokines, and prostaglandins, macrophages can become more aggressive in their anti-inflammation activity (M1) or adopt a suppressor-like character (M2) [123]. There is a growing body of evidence that in pregnancy, intrauterine macrophages express an M2-type phenotype and participate in the Th2 polarization at the feto-maternal interface, via their antigen-presenting ability or secretion of Th2-type cytokines [131]. Human and murine macrophages have been shown to respond to PSG treatment by secreting specific cytokines and by inducing arginase

expression [18] [19] [58]. Treatment of BALB/c primary macrophages and RAW 264.7 with the N1 domain of PSG17N induces the induction of IL-10, IL-6, and TGFβ_1 in a dose-dependent manner. The up-regulation of these cytokines was also observed when we treated thioglycollate-elicited peritoneal macrophages from a Th1-dominant mouse strain, C57BL/6, with PSG17N.

The derivation of J2 transformed bone marrow derived macrophages (BMDM) allowed us to study their responses to PSG17N over a range of concentrations and time points. Our results showed that PSG17N induced the secretion of IL-10, IL-6 and TGFβ_1 in primary macrophages and BMDM of wild type mice, but not in CD9-deficient ones. Such unresponsiveness of CD9KO macrophages to PSG17N is not affected by the increase in PSG17 concentration. These findings implied that CD9 expression is required for PSG17N mediated cytokine induction in macrophages.

CD9 is expressed abundantly in macrophages, DCs, a subset of B cells and T cells. Since CD9 can act as an organizer for the formation of a tetraspanin microdomain, the absence of its expression may affect the presentation, conformation, and density of their associated molecules. Observations of cellular activities, however, revealed almost no impact of the genetic deletion of CD9, except a defective gamete fusion, which is mediated by CD9 expressed on eggs [224]. It is therefore suggested that other tetraspanins might function to compensate for the absence of CD9. This view was put forward in the study of Kaji and coworkers. Injection the CD9-deficient eggs with mRNA of human CD9 or mCD81 can reverse the infertility of these eggs, implicating a potential role for mCD81 in compensating for CD9 dysfunction. However, our research showed that wild type and CD81-deficient macrophages responded similarly to PSG17N

treatment in the secretion of cytokines, revealing that CD9 is the only biological receptor for PSG17N. We also showed that the absence of CD9 or CD81 does not have an impact on macrophage function in producing IL-10, IL-6, and IL-12 in response to LPS stimulation, nor on their ability to up-regulate the arginase activity induced by IL-4 treatment (data not shown).

2.3 CD9 is the receptor for murine PSG19.

The 108-amino acid sequences of N1 domain between PSG17 and PSG19 are highly homologous (in **boldface**) as shown below:

PSG17N1
RVTV**E**F L**PP**QV**VEGEN VLLRVDNLPE NL**LGFV**WY**K**G V**ASM**KLGIAL YSL**QY**NVS**VT G**LK**HSGRETL HR**NGS**LWIQ**N VT**S**EDTGYYT** LR**T**V**S**QR**G**EL VS**D**TS**I**FLQV YS**

PSG19N1
RVTIE**S** V**PP**KL**VEGEN VLLRVDNLPE NL**RVFA**WY**R**G V**IKFK**LGIAL YSL**D**Y**NTS**VT G**PE**HSGRETL HS**NGS**LWIQS A**TR**EDTGYYT** FQ**TI**S**KN**G**KV VS**NTS**M**FLQV YS**

[65].

Both of these N1 domains have 3 potential N-linked glycosylation sites (in **shade**), which contain the conserved sequence Asn-X-Ser/Thr (N-X-S/T, while X can be any residue except proline). The fact that in the nonglycosylated state, PSG17 is unable to bind to CD9 indicates an important role for glycosylation in PSG-receptor interaction (Dveksler, private communication). Furthermore, since PSG17 and 19N induce cytokine secretion in macrophages, it is possible that they both use CD9 as the common receptor.

Using FACS and ELISA we could not confirm that PSG19N binds to CD9 expressing HEK. However, by applying the panning method used for the identification CD9 as the PSG17 receptor, we found that CD9 was also the receptor for PSG19. The

fact that PSG17 binds to CD9 in ELISA and FACS assay while PSG19 does not, might possibly be explained by PSG19's weaker affinity for CD9. Furthermore, PSG19's binding to the CD9-EC2 WT but not to the CD9-EC2-TM, as revealed by the pull-down assays, indicates that a similar mechanism is shared by PSG17 and PSG19, in which both induce a specific set of cytokines in macrophages upon binding to the same residues of CD9.

Our preliminary findings also suggest that CD9 is potentially the receptor for murine PSG18. In a competition assay, preincubation with AP-PSG18 (alkaline phosphate) reduced the binding of AP-PSG17 to macrophages (Dveksler, personal communication). Taken together, these findings support the essential role of CD9 in pregnancy as a receptor for multiple murine PSGs.

2.4 Macrophages induce IL-10, IL-6, and TGFβ_1 in response to PSG17.

As described in detail in the introduction, IL-10, IL-6, and TGFβ_1 have been shown to create a Th2 environment favorable to pregnancy and fetal tolerance. PSG17N behaves like human PSG 1, 6, and 11, inducing macrophages to secrete IL-10, IL-6, and TGFβ_1 in a dose response and time specific manner. Treatment of primary peritoneal macrophages with PSG17N results in the secretion of TGFβ_1 at 2 hours post treatment, whereas the release of IL-10 and IL-6 occurs later, at 6 hours post treatment. In particular, TGFβ_1 has been shown to up-regulate IL-10 and IL-6 in various cell lines including macrophages, peripheral mononuclear cells, and T cell [225-228]. Remarkably, there is evidence that IL-10 and TGFβ_1 can collaborate to promote immune tolerance privileges in some parts of the human body such as the eye [179], or to regulate T cell response in organ transplantation and specific immunotherapy for airborne allergy [170]

[229]. Hence, we decided to investigate the effect of PSG17N-mediated secretion of $TGF\beta_1$ on IL-10 and IL-6 production. TGFβ neutralization is among the widely accepted methods to determine its role in regulating activity of its downstream targets both *in vitro* and *in vivo* [229] [230]. The concentration of anti-TGFβ mAbs used in our assay was in the effective range of the manufacturer-prescribed dose for neutralizing the amount of TGFβ which macrophages produce in response to PSG17N. However, we were unable to demonstrate, via neutralization of TGFβ during PSG17N treatment, that $TGF\beta_1$ secretion results in the up-regulation of IL-10 and IL-6.

2.5 PSG17N induces COX-2 induction and PGE_2 elevation upon binding to CD9.

Prostaglandin (PGE_2) has been shown to play a role in regulating the balance of Th1 and Th2-type response via the modulation of proliferation, activation, apoptosis and cytokine secretion of immune cells [231] [232] [214]. Furthermore, the immunosuppressive properties of PGE_2 include its ability to induce T cell anergy to specific antigens [233] and to down-regulate dendritic cell functions [234]. The essential role of PGE_2 as well as COX-2 in pregnancy is evident in studies of COX-2 deficient mice and of prostaglandin supplementation. [215-217] [205-209].

Since COX-2 and PGE_2 play important roles in reproduction, we decided to investigate whether PSG17N can induce PGE_2 secretion and, as a result, has the ability to potentially regulate both the innate and acquired immune responses. Our results showed that macrophages isolated from wild type but not from CD9-deficient mice released PGE_2 as early as 4 hours post treatment with PSG17N, whereas the accumulation of COX-2 protein in the cell lysates could be detected at 2 hours post treatment. Furthermore, we determined that the elevation of PGE_2 was the result of COX-2 induction because the

cotreatment of macrophages with PSG17 and a COX-2 specific inhibitor, NS-398, reduced the production of PGE_2. Both wild type and CD9-deficient macrophages responded equally to treatment with LPS alone or with LPS plus COX-2 inhibitor**,** indicating that the lack of CD9 expression did not cause any defect in the pathway.

2.6 COX-2 mediated PGE_2 and cAMP-dependent PKA are involved in the up-regulation of IL-10 and IL-6.

Starting from our discovery that IL-10 and IL-6 up-regulation in macrophages in response to PSG17N requires *de novo* protein synthesis, we took more steps to look at signaling pathways that might regulate the gene expression of IL-10 and IL-6, with PGE_2 being our first candidate. PGE_2 has been reported to regulate IL-6 and IL-10 production in activated macrophages [232] [235]. The engagement of PGE_2 to its subtypes of G-protein-coupled receptors, EP2 or EP4, which are expressed predominantly in macrophages, leads to the alteration in expression of specific genes (Figure 6B). This process is carried out mainly through the activation of protein kinase A (PKA) that takes place when there is an intracellular accumulation of cAMP. Via its binding to the PKA regulatory subunits, this second messenger mediates the release of the catalytic subunits, which later relocate to the nucleus and phosphorylate the transcription factors, such as CREB, initiating gene transcription. (Figure 7)

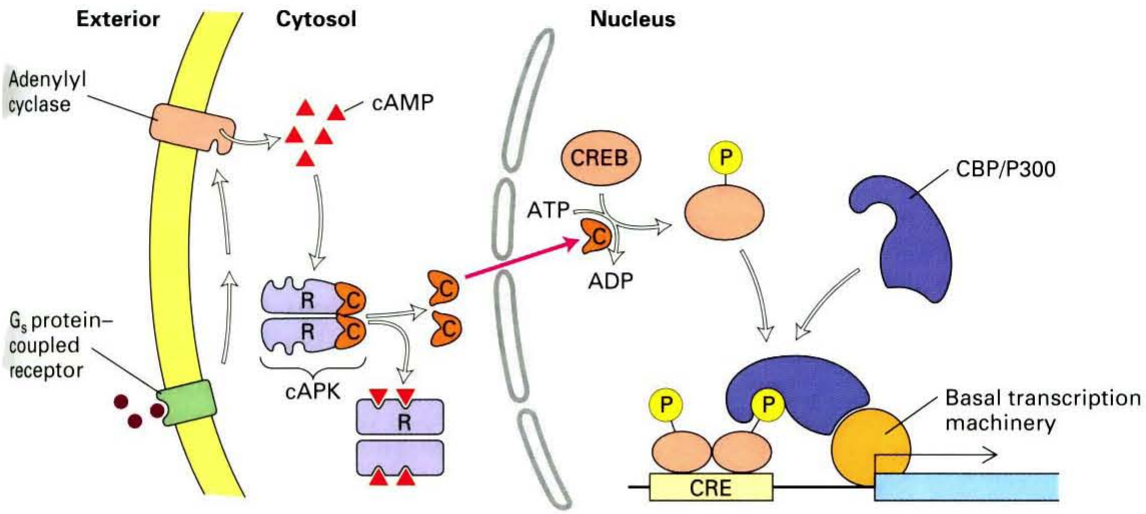

Figure 7. **cAMP-dependent signaling pathway activating transcription factors and modulating gene expression following ligand binding to G_s protein-linked receptor.** The binding of PGE_2 to EP2 or EP4 activates adenylcyclase, which in turn synthesizes cAMP. Upon the binding of cAMP to the regulatory subunits of holoenzyme PKA (cAPK), the catalytic subunits are released and translocated to nucleus, where they phosphorylate CREB (cAMP- response element binding protein) at Ser133. By interacting with CBP (CREB binding protein)/P300, CREB mediates the transcription of CRE-containing genes (including COX-2, IL-10, and IL-6…). (Source: Lodish et al., *Molecular Cell Biology*, 2000, p. 903)

The finding that the introduction of NS-398 greatly reduced the level of IL-6 and IL-10 in macrophages treated with PSG17N prompted us next to study the involvement of the cAMP-dependent PKA pathway in the PSG17N-mediated induction of IL-10 and IL-6. While we could not detect an elevation of intracellular cAMP in response to PSG17N treatment, we found that the introduction of the cAMP-dependent PKA selective inhibitor, KT5720, significantly reduced the secretion of IL-10 and IL-6. Recently, Baker provided evidence that a weak agonist of G-protein-coupled receptors, while failing to elevate intracellular cAMP level, still efficiently induced cAMP-mediated gene regulation. In this case, a prolonged and sustained turnover of cAMP seems sufficient to cause a sustained phosphorylation of its downstream target, CREB, and eventually CRE-gene transcriptional activation [236]. Since KT5720 competes with cAMP for binding to the regulatory subunits of PKA, its effect in reducing PSG17N mediated IL-6 and IL-10 synthesis, therefore, indicates that an activation of cAMP-dependent PKA has occurred.

cAMP-dependent PKA phosphorylation of ser133 on CREB is essential for this transcription factor to bind to its co-activator CBP/p300, which in turn, in association with the transcription machinery, initiates target gene expression [237] (Figure 7). Most of the genes regulated by cAMP-dependent PKA contain a cis-acting DNA sequence, named cAMP-responsive element (CRE), which is the binding site for CREB. Interestingly, the COX-2, IL-10, and IL-6 genes have CRE sites in their 5' flanking promoter region [238] [239] [240] [241]. Our preliminary experiments to examine the effect of PSG17N on CREB phosphorylation, showed that phosphorylated CREB could be detected 15 min post PSG17N treatment only in wild-type but not in CD9-deficient

BMDM. Future studies are needed to determine the duration and magnitude of the phosphorylation of CREB as well as the definitive role of CREB in IL-10 and IL-6 up-regulation.

While the mechanisms of TGF signaling in cellular activities and mammal development are well established, the regulation of $TGF\beta_1$ expression is still poorly defined [242]. In cell types other than macrophages, protein kinase C and extracellular signal regulated kinase (ERK) are involved in the up-regulation of $TGF\beta_1$ [243] [244]. Our experiment studying mRNA of $TGF\beta_1$ reveals no change in the level of $TGF\beta_1$ transcript at 2 hours post treatment with PSG18 compared to the control treated group. We also found that the PSG17N mediated secretion of $TGF\beta_1$ in macrophages is not regulated by the COX-2/PGE_2 and cAMP-dependent PKA pathway.

2.7 A proposed model for the signaling pathway in the PSG17-CD9 mediated up-regulation of IL-10 and IL-6

On the basis of the findings presented earlier, we propose a signaling pathway that underlies the PSG17 mediated cytokine induction, as shown in the following diagram (Figure 8): The binding of PSG17 to the large extracellular loop of CD9 modifies the lateral interaction between CD9 and signaling molecules. Activation of the unknown signaling mechanism(s) induces the expression of COX-2, which subsequently leads to the secretion of PGE_2. The upregulation of IL-10 and IL-6 is partially mediated by the PGE_2 elevation itself (Pathway I) and by the activation of the cAMP-dependent PKA pathway (Pathway II). While these pathways do not participate in the induction of $TGF\beta_1$ release, we speculate that PKC or MAPK plays a role in $TGF\beta_1$ upregulation and that these two signaling molecules might take part in the induction of IL-10 and IL-6.

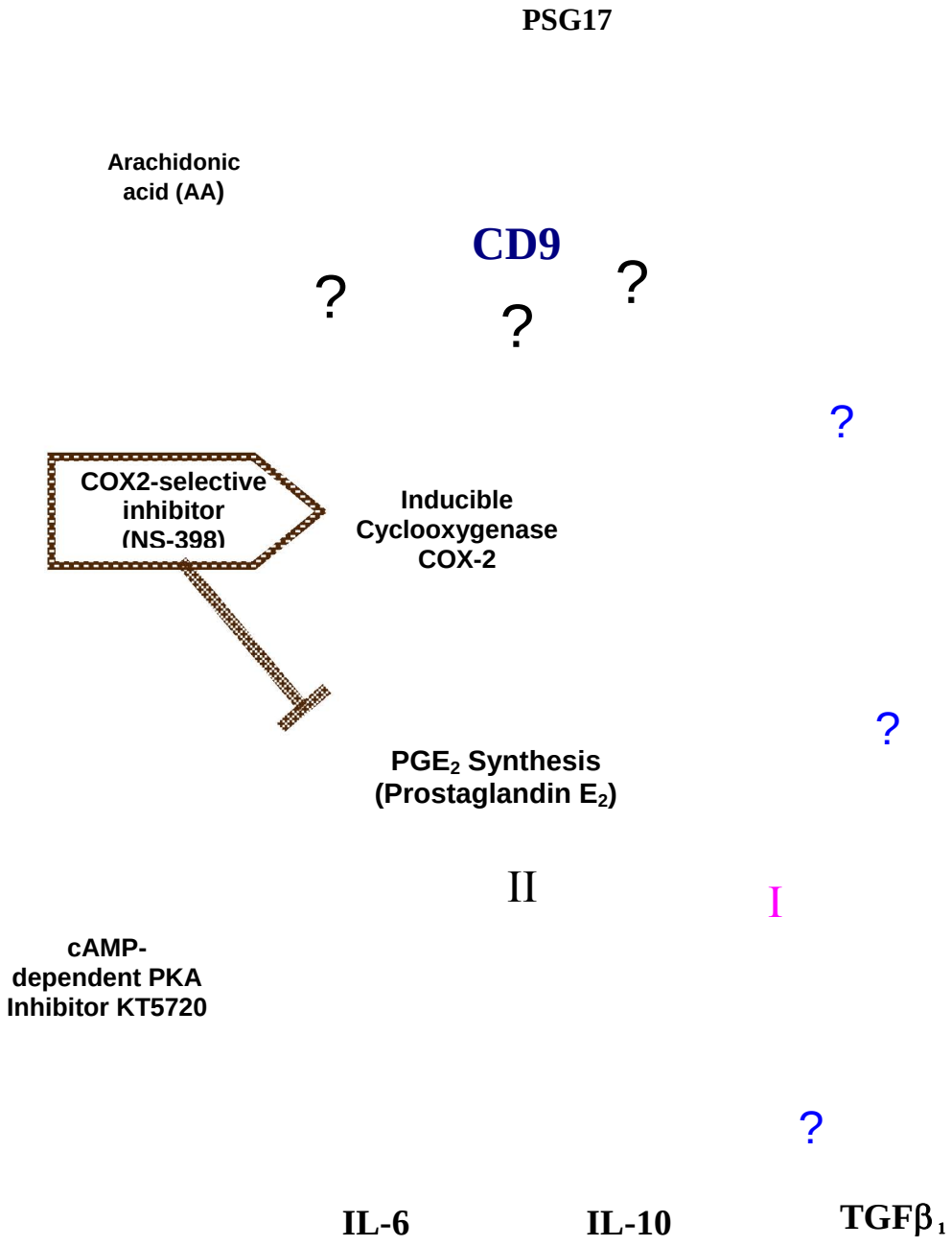

Figure 8. Proposed signaling pathway for IL-10 and IL-6 upregulation resulting from binding of PSG17N to CD9

3 FUTURE DIRECTIONS

3.1 Examination of the involvement of CD9 individual domain in PSG17-mediated cytokine induction

While our present research demonstrated that the large extracellular loop of CD9 is the binding site for PSG17, it remains unclear how the signaling cascade of cytokine induction is initiated and modulated upon the CD9-PSG17 binding. We are therefore, interested in investigating the involvement of other CD9 domains and of the laterally interacting molecules in this process.

CD9 domains, other than the EC2, have several remarkable features that hint at their ability to recruit signaling or stabilize the lateral interaction of CD9 with other associated molecules. For instance, Zhang et al. demonstrated that CD9 and other tetraspanins mediate integrin β_1 function by linking protein kinase C (PKC) to the proximity of β1-containing integrin molecules. In this example, the translocation of cytoplasmic PKC to the cell surface results from an interaction between PKC and the cytoplasmic tails and/or the transmembrane domain 1 and 2 of CD9 [91]. A molecule that has attracted considerable interest is EWI-F, a member of the type I transmembrane family of proteins in the Ig superfamily. EWI-F is the most robust partner of CD9 in macrophages. Recent functional studies of EWI members (EWI-F, EWI-2, EWI-3, and CD101) suggest their involvement in immune-regulation. This includes the down-regulation of prostaglandin F2α receptor by EWI-F [87], the inhibition of TCR/CD3-induced IL-2 production [245], and IL-10-mediated suppression of T cell proliferation by CD101 [246]. The high stoichiometry of EWI-F and CD9 in the complex, its expression in macrophages, and its potential in signaling make EWI-F a candidate for CD9-

associated signaling. In addition, CD9 molecules are subjected to palmitoylation, a process that is required for the translocation of certain signaling molecules to the glycolipid-enriched microdomain (GEM) of cell membrane as well as for their phosphorylation, as seen in LAT, an adaptor molecule in T and B cell activation pathways [247]. Interestingly, site-directed mutagenesis studies have shown that any of the four transmembrane cysteine residues of CD9 adjacent to the cytoplasmic side can be palmitoylated. Mutation of all juxta-membrane cysteine residues (CD9 ^{plm}) causes an instability of CD9-tetraspanin interaction but does not affect the ability of CD9 to translocate to particular microdomains [248] (Figure 9 A and B).

On the basis of these findings, we hypothesize that binding of PSG17N to the extracellular loop 2 of CD9 modulates the lateral interaction of CD9 with its associated partners and results in the recruitment of signaling molecules, leading to the observed induction of cytokine secretion. To test this hypothesis we will examine the contribution of individual domains of CD9 in cytokine induction mediated by PSG17. The effect of the mutations in the cytoplasmic tail and in the palmitoylated cystein as well as the results of the replacement of CD9 transmembrane domains with the corresponding domains of other tetraspanins will be examined in CD9-deficient macrophages expressing these CD9 mutants. In our preliminary experiments, we determined that CD9-deficient macrophages are able to express ectopic proteins like GFP when they are infected with GFP-encoding Lentiviruses. By evaluating the cytokines released upon PSG17 treatment of macrophages expressing CD9 mutants, we will be able to determine the domains that contribute to the process. This will also allow us to identify putative CD9 partners,

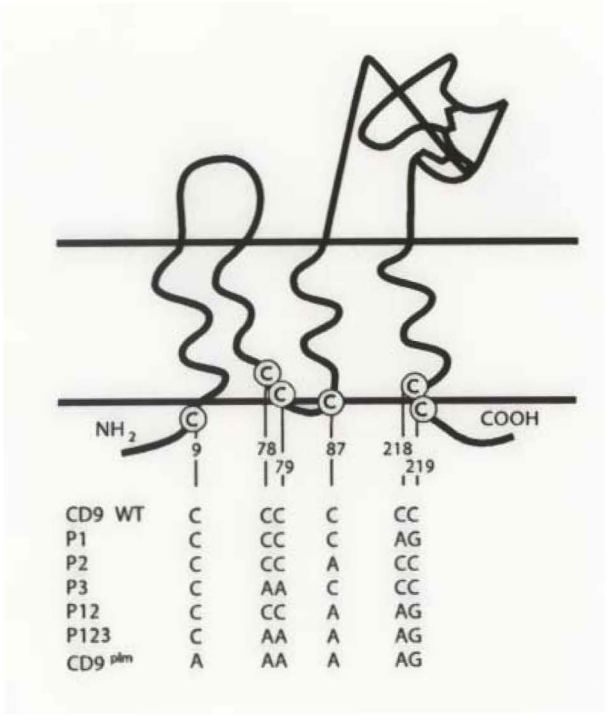

Figure 9A. Representation of CD9 with indication of the internal juxtamembrane cystein residues, and their change in the mutant molecules (Source: Charrine et al., *FEBS Letters*, 516: 139-144, 2002)

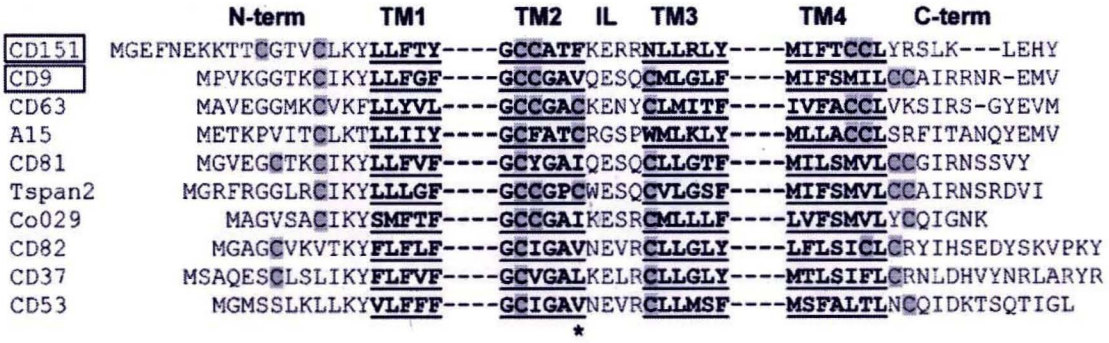

Figure 9B. Mutation of palmitoylation sites in CD151 and CD9 establishes that six membrane-proximal cysteine residues in each molecule can be palmitoylated. It is assumed other conserved cysteine residues (in gray) of tetraspanins are also palmitoylated. (Source: Stipp et al., *TRENDS in Biochemical Sciences*, 28:106-112, 2003)

known to associate with specific domains of CD9, which participates in the PSG17-triggered response.

3.2 Investigation of the involvement of Syk, PKC, and the mitogen activated protein kinase (MAPK) family in the response to PSG17

The tyrosine kinase Syk functions in many immunoreceptor signaling events in various hemapoietic cells, including proliferation, differentiation, and phagocytosis [249]. In addition, Syk activation, which is associated with integrin receptor-ligand interaction, is believed to be essential for hemapoietic cells to attach to the extracellular matrix, an event needed for their proliferation and differentiation [250] [251]. The co-cross-linking of CD9-Fc gamma receptor that is induced by the anti-CD9 antibody KMC8.8 on macrophages leads to Syk phosphorylation and macrophage activation [88]. It is therefore suggested that PSG17 upon binding to CD9 might be involved in the activation of Syk and macrophage as well.

PKC and MAPK activation may be implicated in the response of macrophage to PSG17. In macrophages, elevated intracellular cAMP results in extracellular signal-regulated kinase (ERK) activation [252]. In addition, Williams and colleagues reported that the synthesis of IL-10, in response to PGE_2, is regulated by p38 mitogen-activated protein kinase (MAPK) in inflammatory macrophages [235]. There is evidence that MAPK family activation correlates with COX-2/PGE2 up-regulation and, as a result, increases the production of IL-6 [253] [254]. Furthermore, Giroux reported that PKC-α modulates COX-2 expression in macrophages exposed to both LPS and IFN-γ although the mechanism by which this occurs was not identified [255]. Interestingly, CD9 has been shown to associate with activated PKC in Jurkat and K562 cell lines [91]. In light of

these findings, the relationship between the MAPK family and PKC, on the one hand, and the activation of PSG17N-mediated COX-2/PGE_2 pathway, which has been shown to be a major regulator of the synthesis of IL-10 and IL-6, on the other, merits investigation.

To test whether these molecules participate in cytokine induction, we will evaluate the response of macrophages to PSG17 in the presence and absence of specific inhibitors.

In addition, the role of CCAAT/enhancer-binding protein (C/EBP-α and -β), a transcription factor for basal and cAMP-simulated IL-10 transcription in monocytic cells [256], in PSG17N-mediated cytokine induction needs to be examined.

3.3 Study of the effect of PSG17 in T cells and dendritic cells

CD9 is expressed abundantly in T cells and dendritic cells and its role has been indicated in the regulation of antigen-presenting function of DCs and of T cell proliferation and activation [92] [82]. Acting as a ligand for CD9, it is possible for PSG17N to regulate these cells directly or indirectly by promoting cytokine release or at the time of antigen-specific stimulation mediated by APC. Our preliminary data show that PSG17N induces IL-10, IL-6, and $TGF\beta_1$ in bone marrow-derived dendritic cells isolated from wild type but not CD9-deficient mice. Meanwhile, it has also been reported that the effect of PSG1a-mediated inhibition of T cell activation is dependent on the presence of APCs, indicating a function for PSGs in both innate and adaptive immune cells [58, 59].

We believe that either PSG17 induced PGE_2 secretion by macrophages or the depletion of L-arginase as a result of arginase I expression in these cells might be

responsible for the PSG-mediated inhibition of T cell proliferation. Experiments are underway to test our hypothesis.

3.4 Cloning receptors for human and for other murine PSGs

Although human and murine PSGs induce a particular set of anti-inflammatory cytokines in macrophages, our preliminary data show that CD9 is not the receptor for human PSGs because treatment of wild type and CD9-deficient macrophages with human PSG 1 and 11 induced a similar response: induction of anti-inflammatory cytokines. Furthermore, testing the binding of human PSGs to various tetraspanins (CD81, CD82, CD53, CD37, CD151, and NAG2) expressed in HEK cells, revealed that human PSGs did not use any of these tetraspanins as their receptors. Therefore we propose to clone the human PSG receptor by panning in cases where the putative receptor is composed of a single polypeptide chain, or by applying affinity chromatography followed by SDS-PAGE and microsequencing for detecting the receptor of multiple polypeptide chains.

It is also of interest to determine whether murine PSGs other than PSG17 and 19 use CD9 as their receptor. In this case, testing the response of CD9-deficient macrophages to the treatment of those PSGs and analyzing the binding of CD9 to them by pull-down will provide us with the answers.

3.5 Characterization of the glycan group in PSG biological functions

Recently, Clark and co-workers hypothesized that developing human and gametes are protected by the soluble glycoproteins called Glycodelin A (GdA) and Glycodelin S (GdS), which are found in amniotic fluid and seminal plasma. The unusual N-linked oligosaccharides of these glycodelin proteins have been suggested to be the glycans that are obtained by the human immunodeficiency virus (HIV) upon their

generation in T cell, and that are used by HIV to disrupt the carbohydrate-dependent immune cell interaction, resulting in an aberrant immune response. In addition, the expression of these glycans in human tumor cells, intravascular helminthic parasites, and *Helicobacter pylori* implies that they might have a role in countering the attack by the immune system [257].

The PSGs are highly glycosylated and possess immuno-modulation functions. However, whether they have the same glycans as the glycodelins and whether the glycosylation of PSGs mediates their immuno-regulating property are questions that need addressing. Because the enzymes responsible for the glycan modification of glycoproteins (glycosidases and glycosyltransferase) are cell specific, we propose to characterize the glycans of human PSGs that are collected from human placenta or synthesized in human trophoblast cell lines.

3.6 Study of the autocrine effect of PSG17 in extravillous trophoblasts

CD9 is highly expressed in human second and trimester extravillous trophoblasts (EVT), which invade the endometrium during implantation and placentation. It has been suggested that CD9 plays a role in uterine invasion together with integrin $\alpha 5\beta 1$ [81, 258]. It is possible that PSGs may interact with CD9 on extravillous trophoblasts and, by this autocrine mechanism, has a direct effect on trophoblast activities. This can be tested by examining the response of trophoblast cell lines or primary EVT to PSG treatment.

* * *

In the final analysis, to gain more understanding of PSG17 function, *in vivo* studies to analyze the systemic effect of PSG17N administration in mice challenged with pathogens or susceptible to autoimmune diseases such as experimental acute encephalitis

(EAE) could be informative. Whether PSGs have an important role systemically or at the placental-maternal interface remains undetermined. Hormones such as progesterone regulate the immune response at locations far from its original site of production but its use for the management of autoimmune disorders is limited. Therefore we propose that administration of PSGs to generate a Th2 environment could be potentially beneficial in Th1-associated diseases. Before this is feasible, further questions on the biology of these proteins outlined in this section need to be addressed.

PART FOUR

REFERENCES FOR INTRODUCTION AND DISCUSSION

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